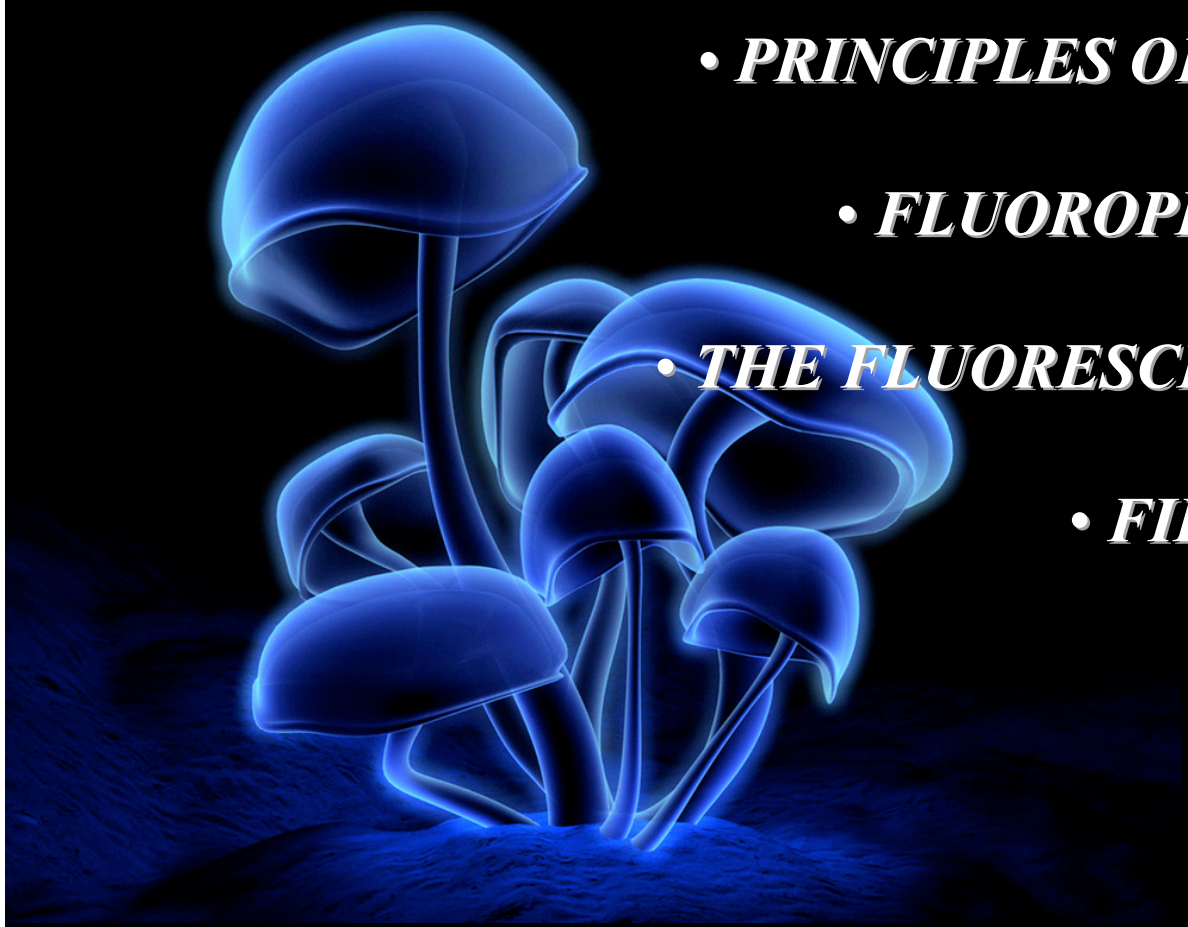
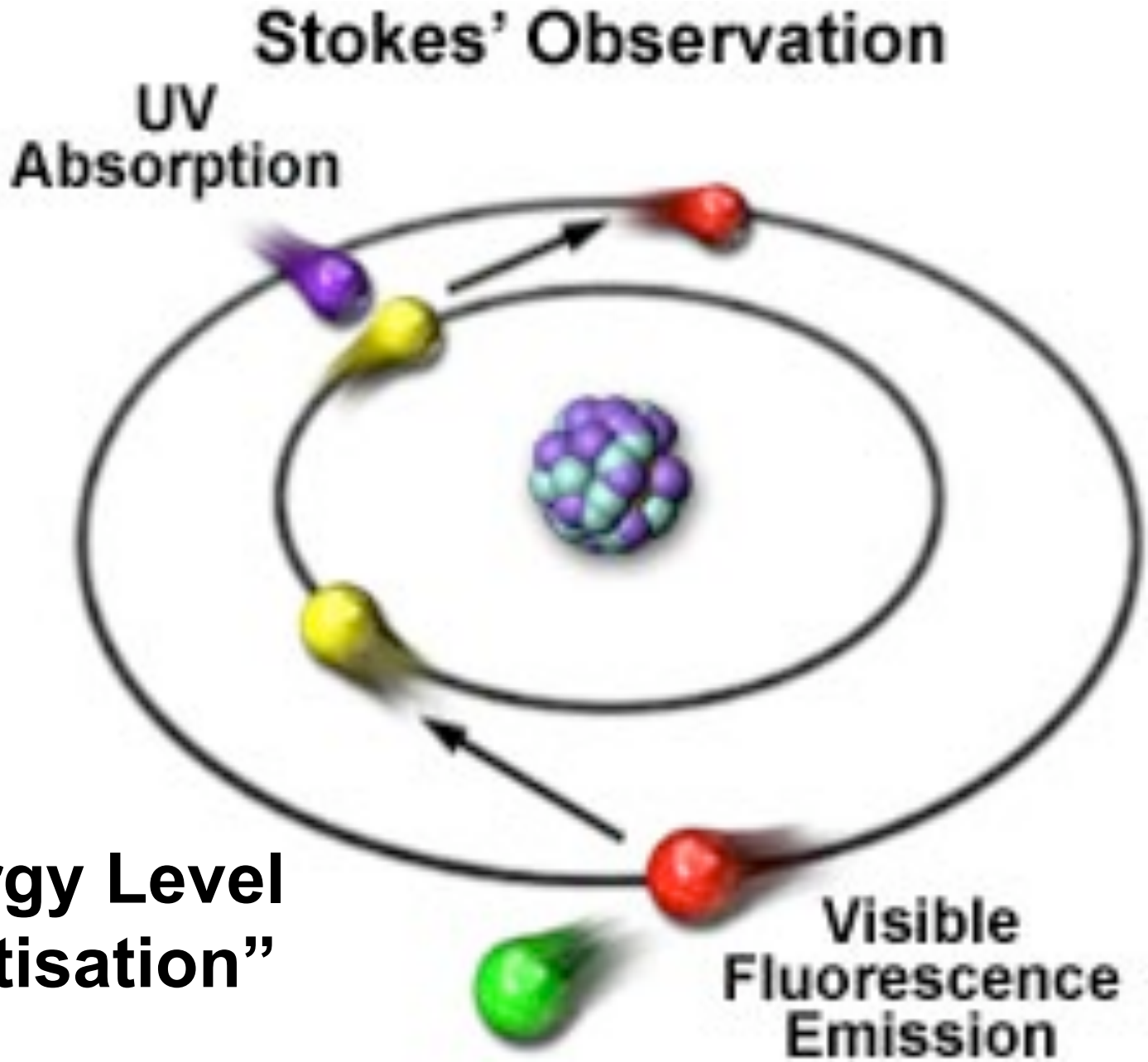
FLUORESCENCE MICROSCOPY

TOPICS



- PRINCIPLES OF FLUORESCENCE
- FLUOROPHORES / DYES
- THE FLUORESCENCE MICROSCOPE
- FILTERS



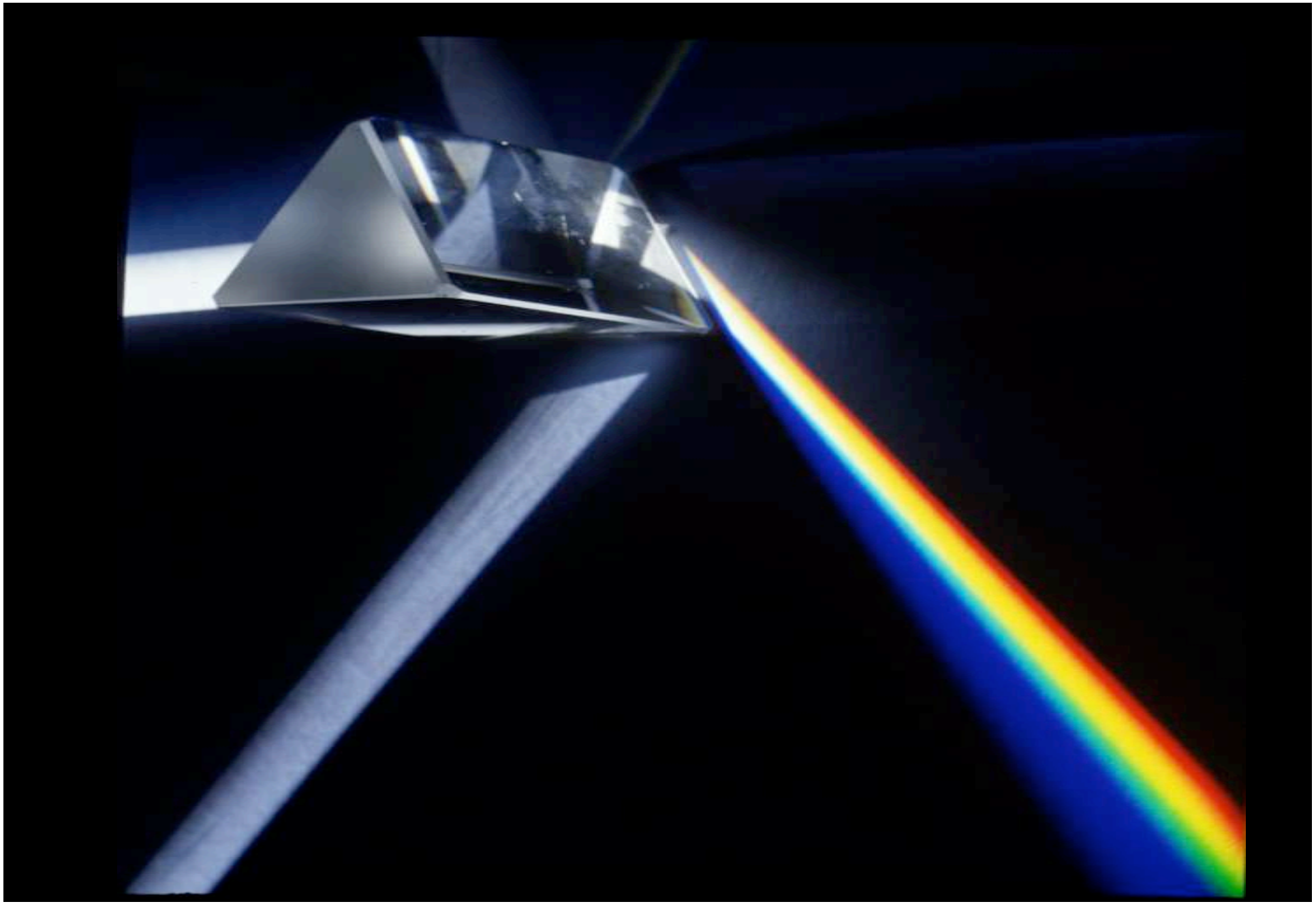
"Energy Level Quantisation"

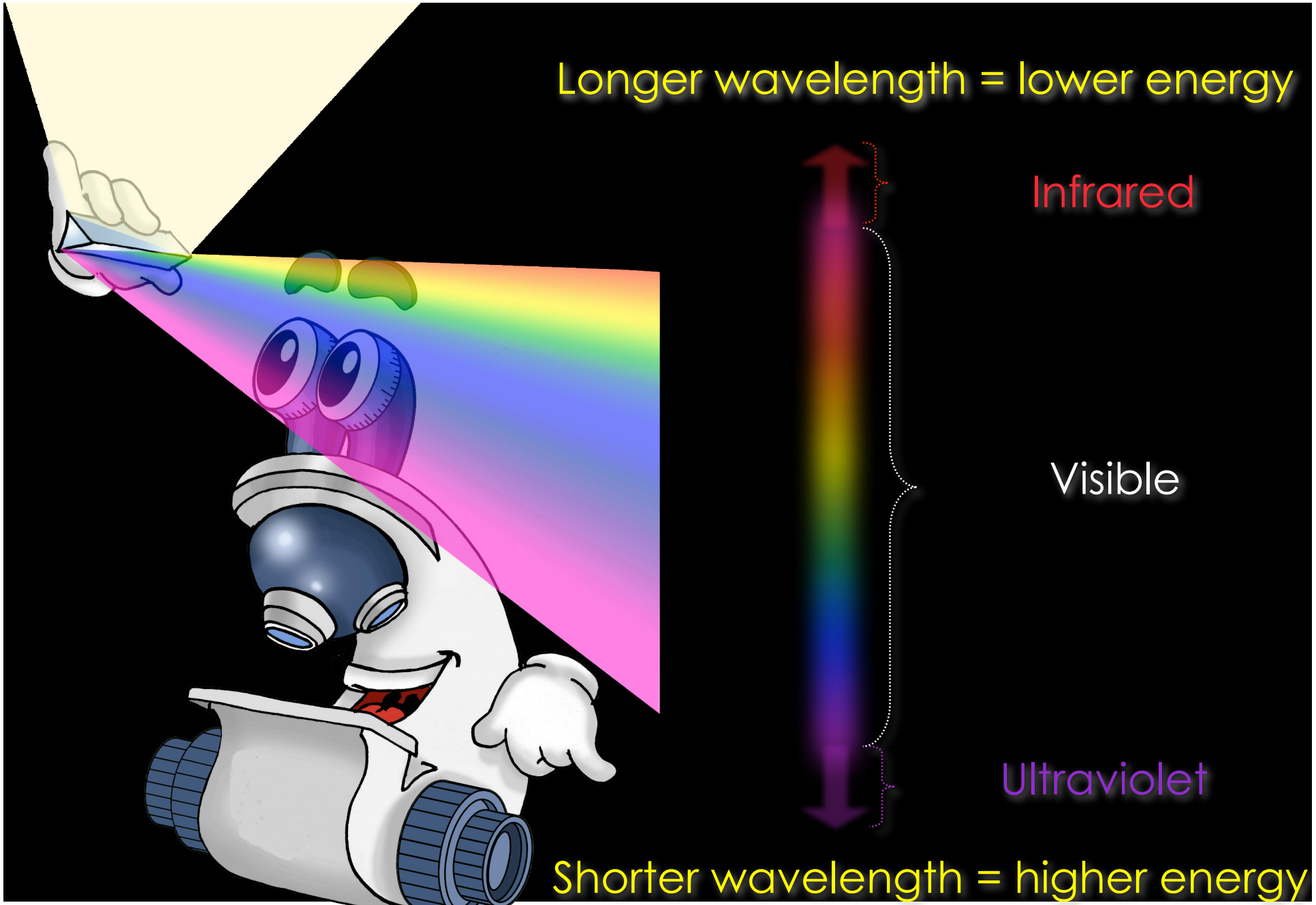
Processes leading to fluorescence in fluorescent dyes

1. **Absorption of a photon by a Dye Molecule**

2. **Dye Molecule relaxes**

3. **Dye Molecule emits a photon**




Longer wavelength = lower energy
Infrared
Visible
Ultraviolet
Shorter wavelength = higher energy

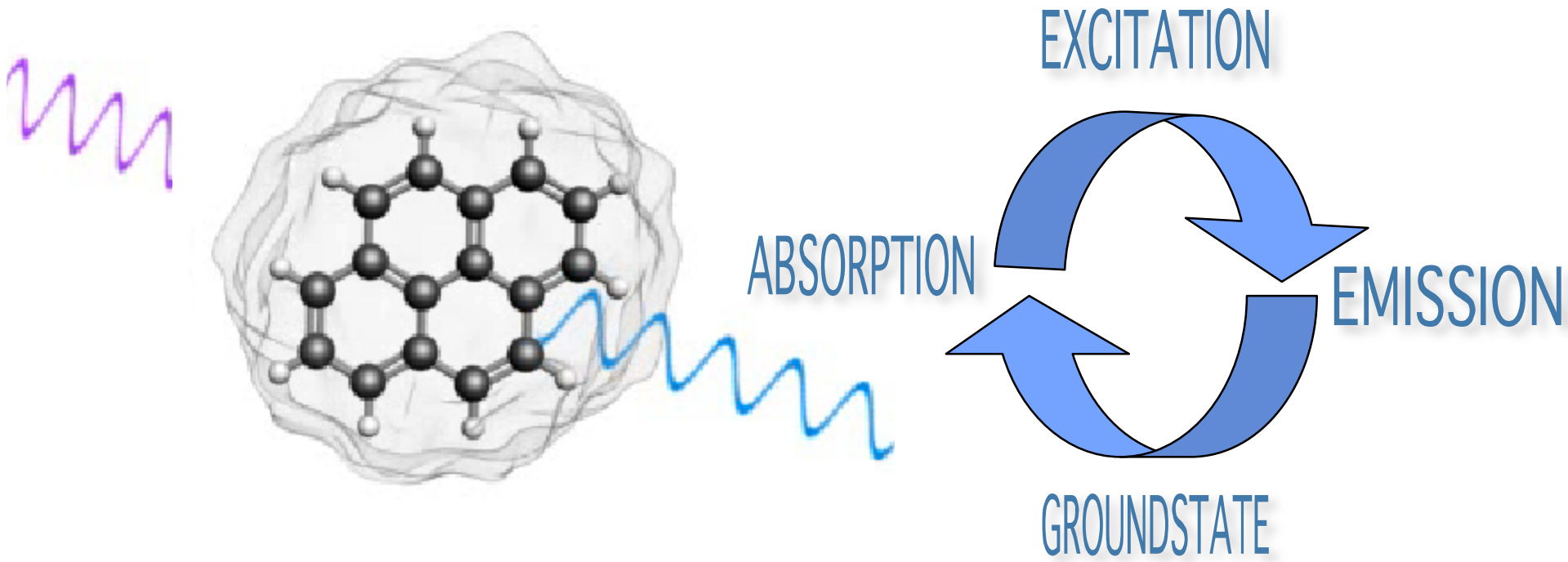
ABSORPTION OF LIGHT

All molecules absorb light
different molecules - different wavelengths

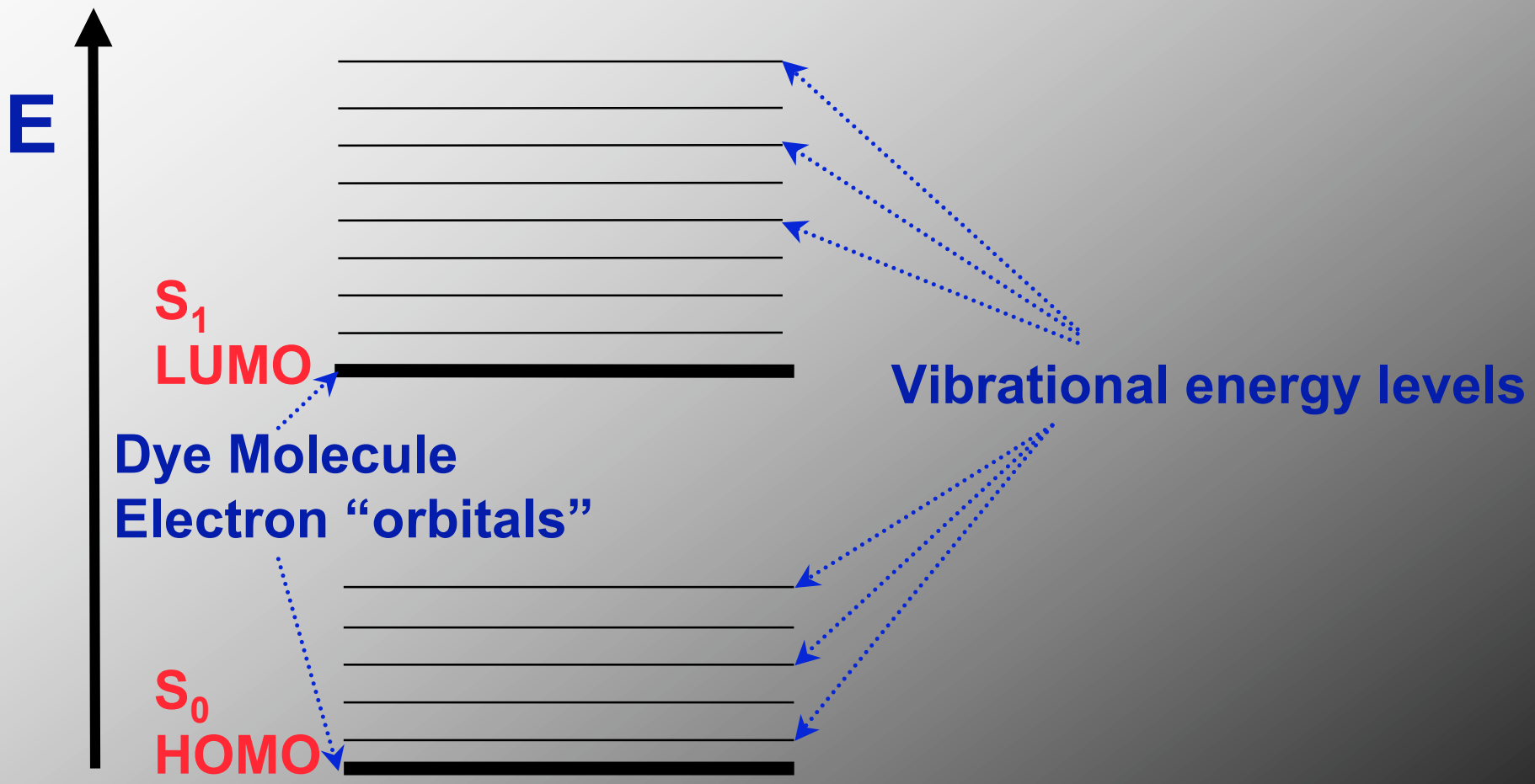
– Absorption of microwaves causes molecular rotations,

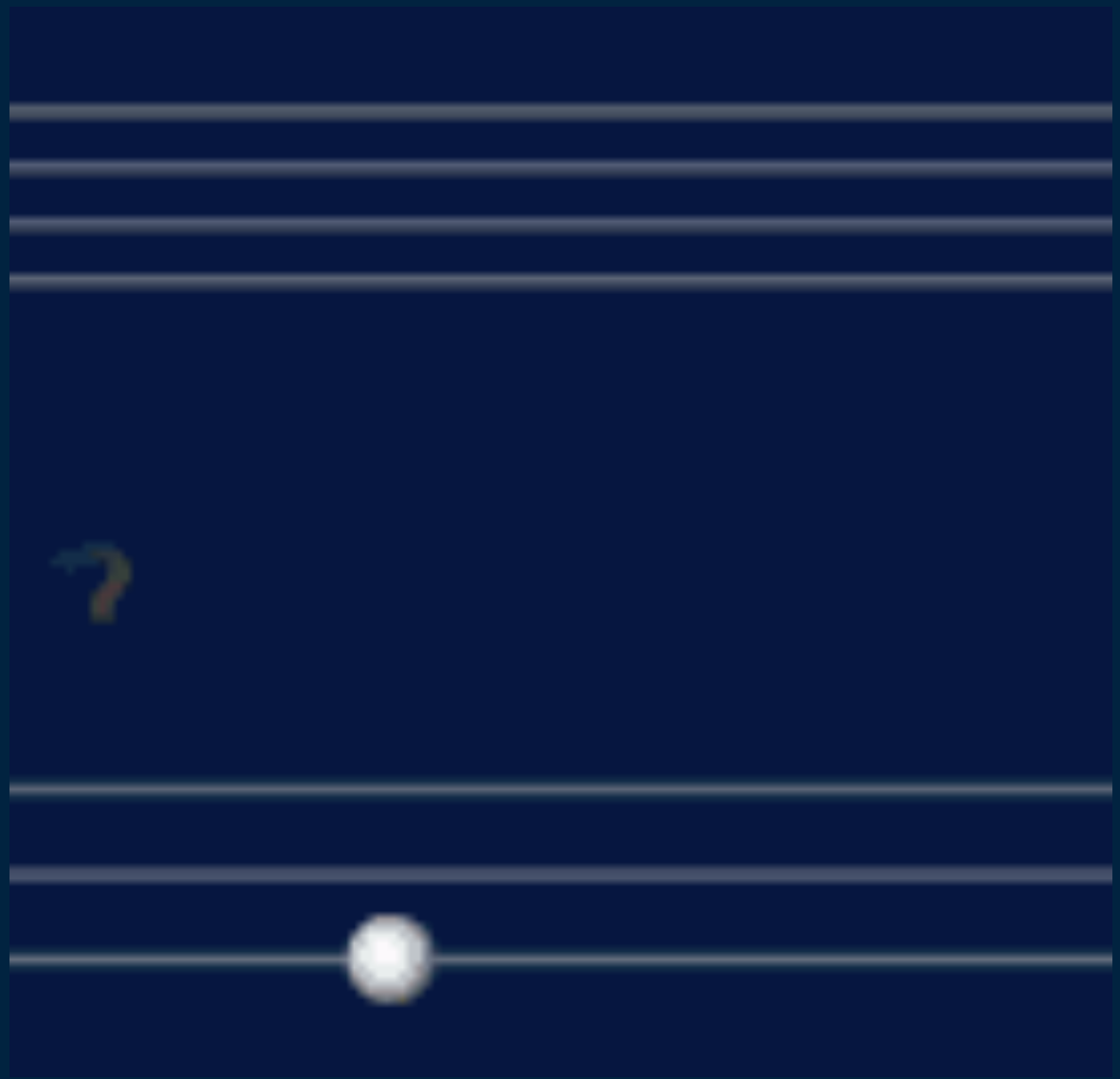
– Absorption in the infra red causes molecular bond vibrations,

– Absorption of UV/visible light causes electrons to jump to higher energy “orbitals”.

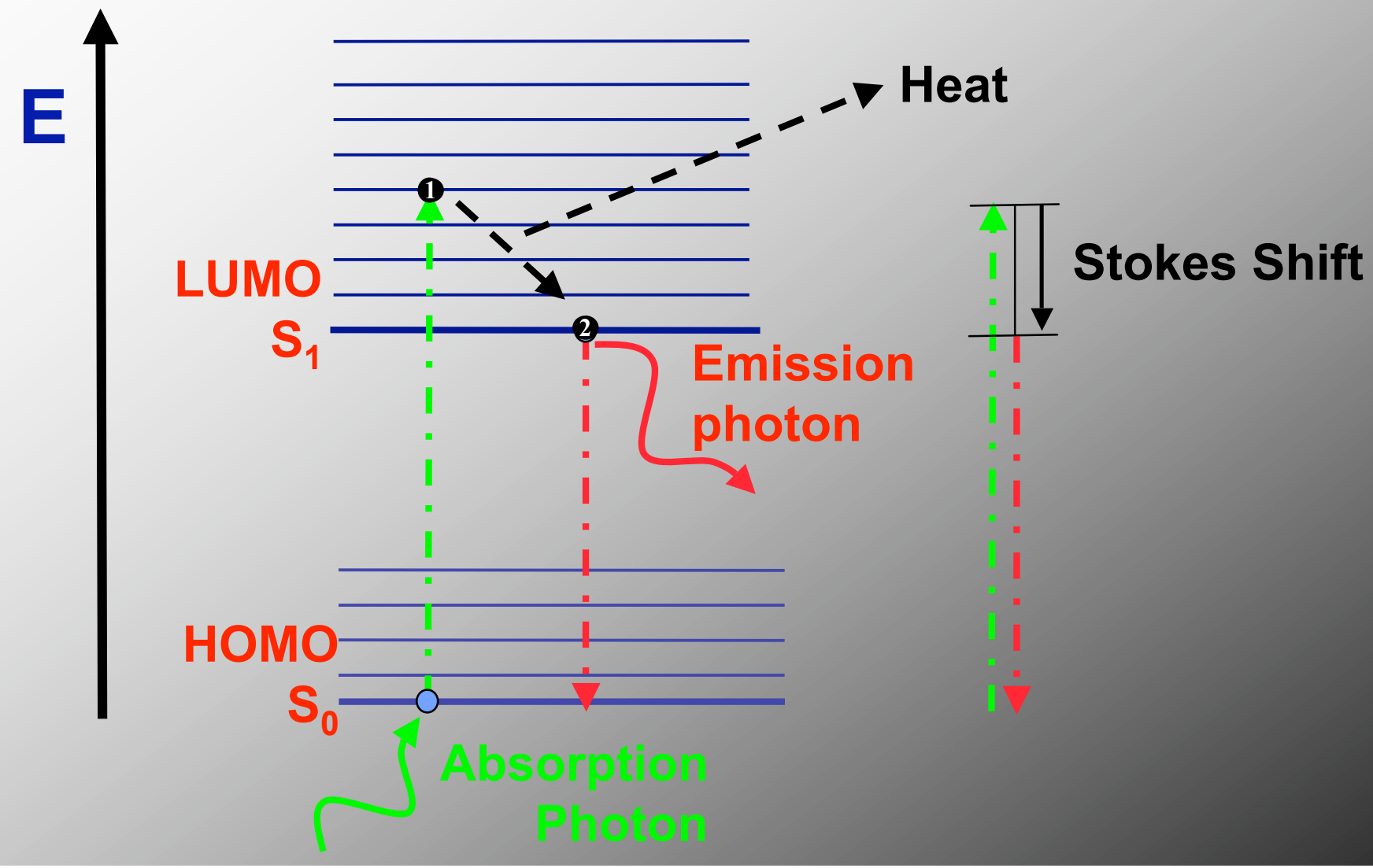

EXCITATION
ABSORPTION
EMISSION
GROUNDSTATE

Energy Level Diagram



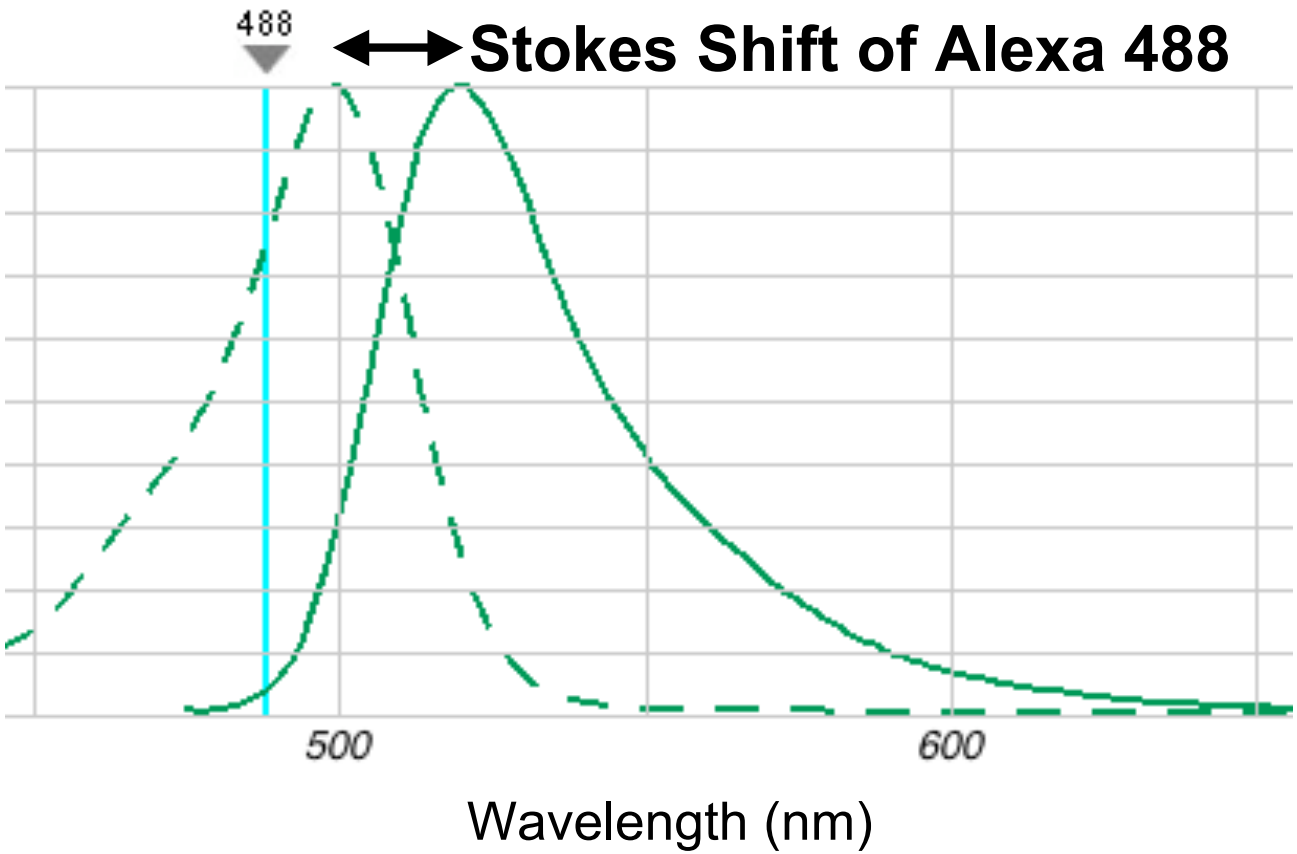


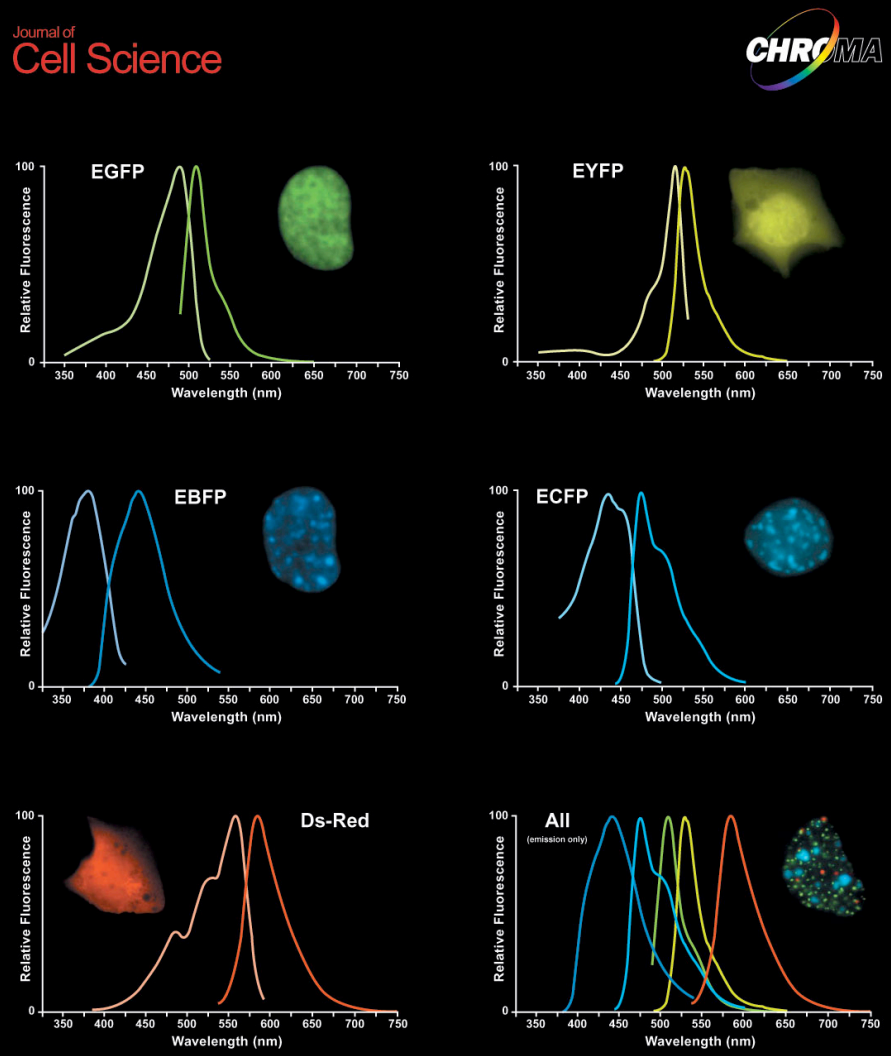
Fluorescence Absorption / Emission



Emission has lower energy ⇨ **Longer wavelength**

Absorption = Excitation Emission = Fluorescence





Fluorescent Protein Spectra

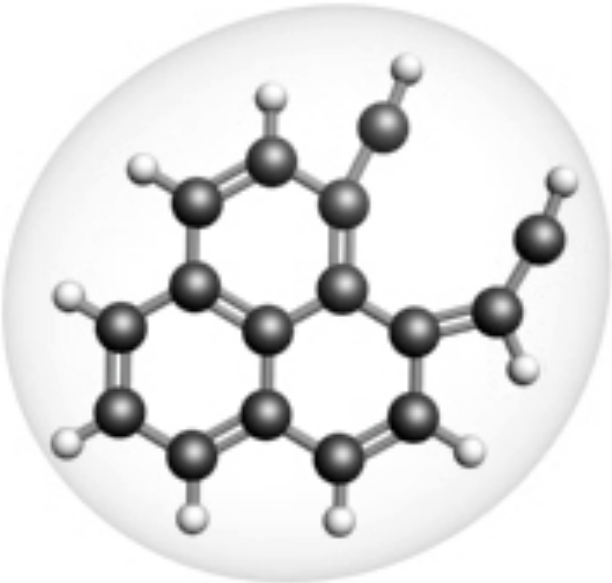
George Patterson, Rich N. Day and David Piston

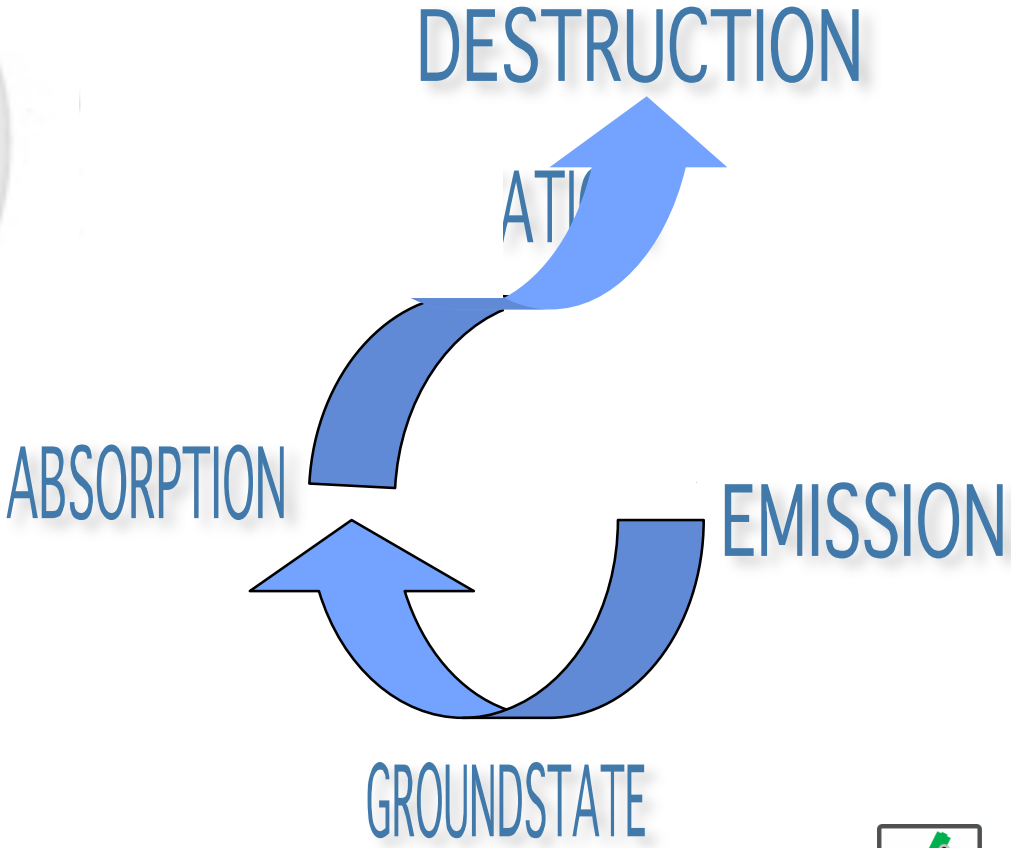
©Journal of Cell Science 2001 (114, pp. 837-838)

1st TAKE HOME MESSAGE.....

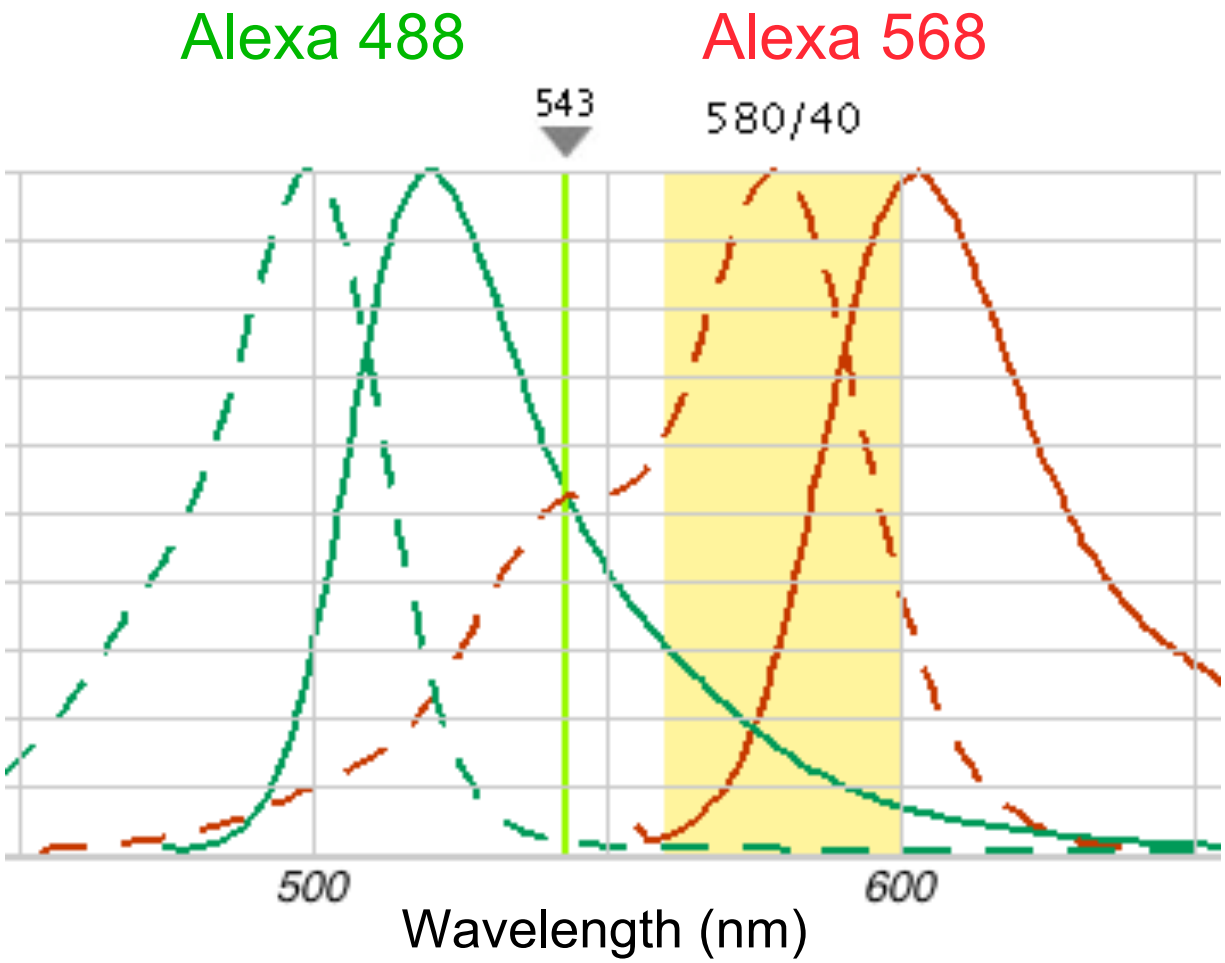
KNOW YOUR FLUOROPHORE!!!!

PHOTOBLEACHING

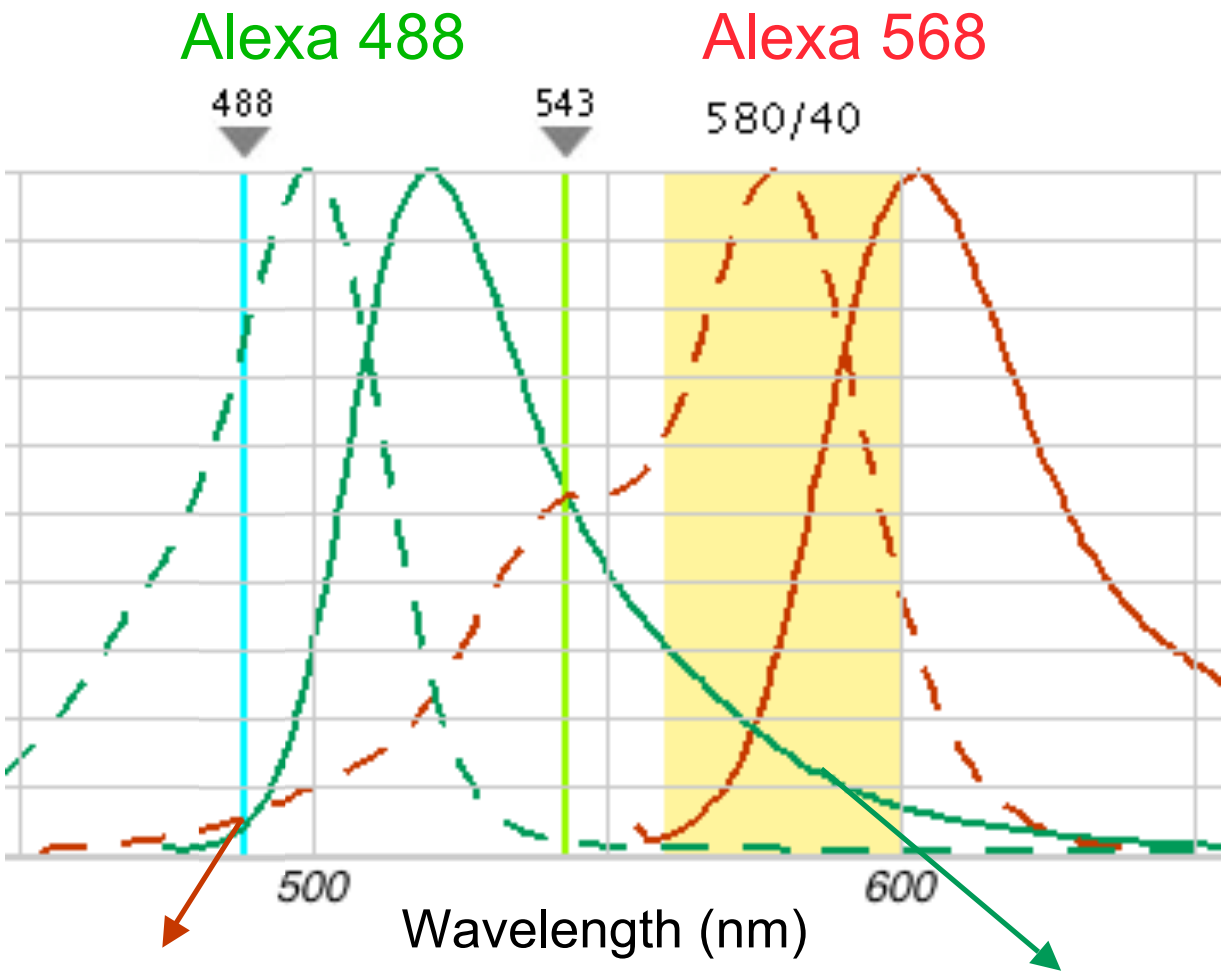




Multiple colour/dye imaging…

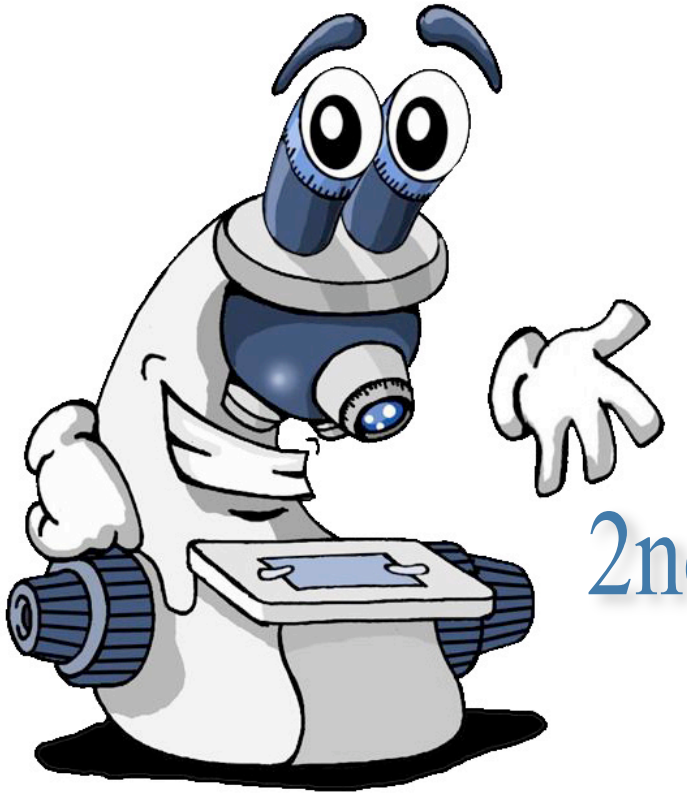


Beware ! Crosstalk and Bleed Through



Cross talk (wrong excitation)

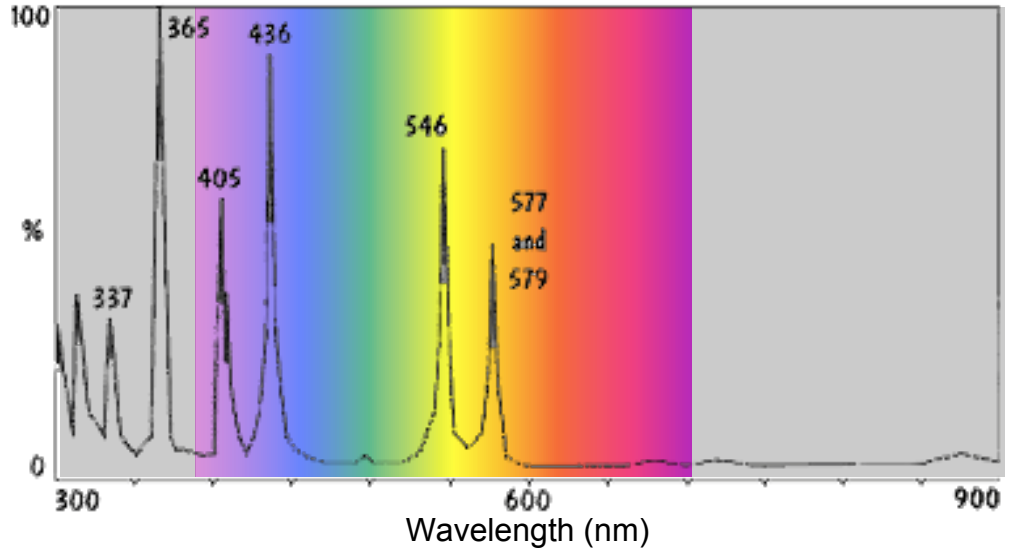
Bleed through (wrong emission)



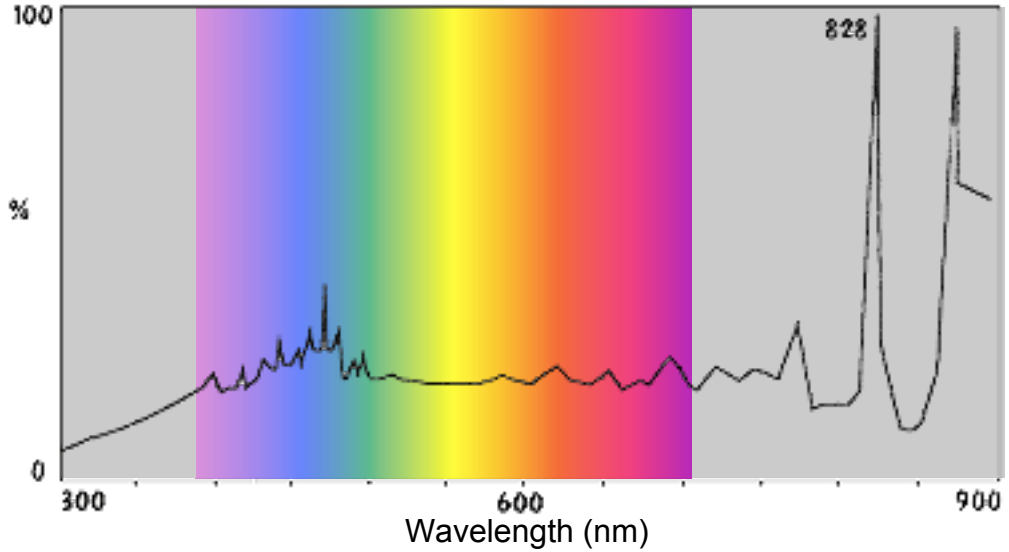
2nd TAKE HOME MESSAGE.....
KNOW YOUR MICROSCOPE!!!!

NO LIGHT.....

Spectrum of a Mercury Lamp

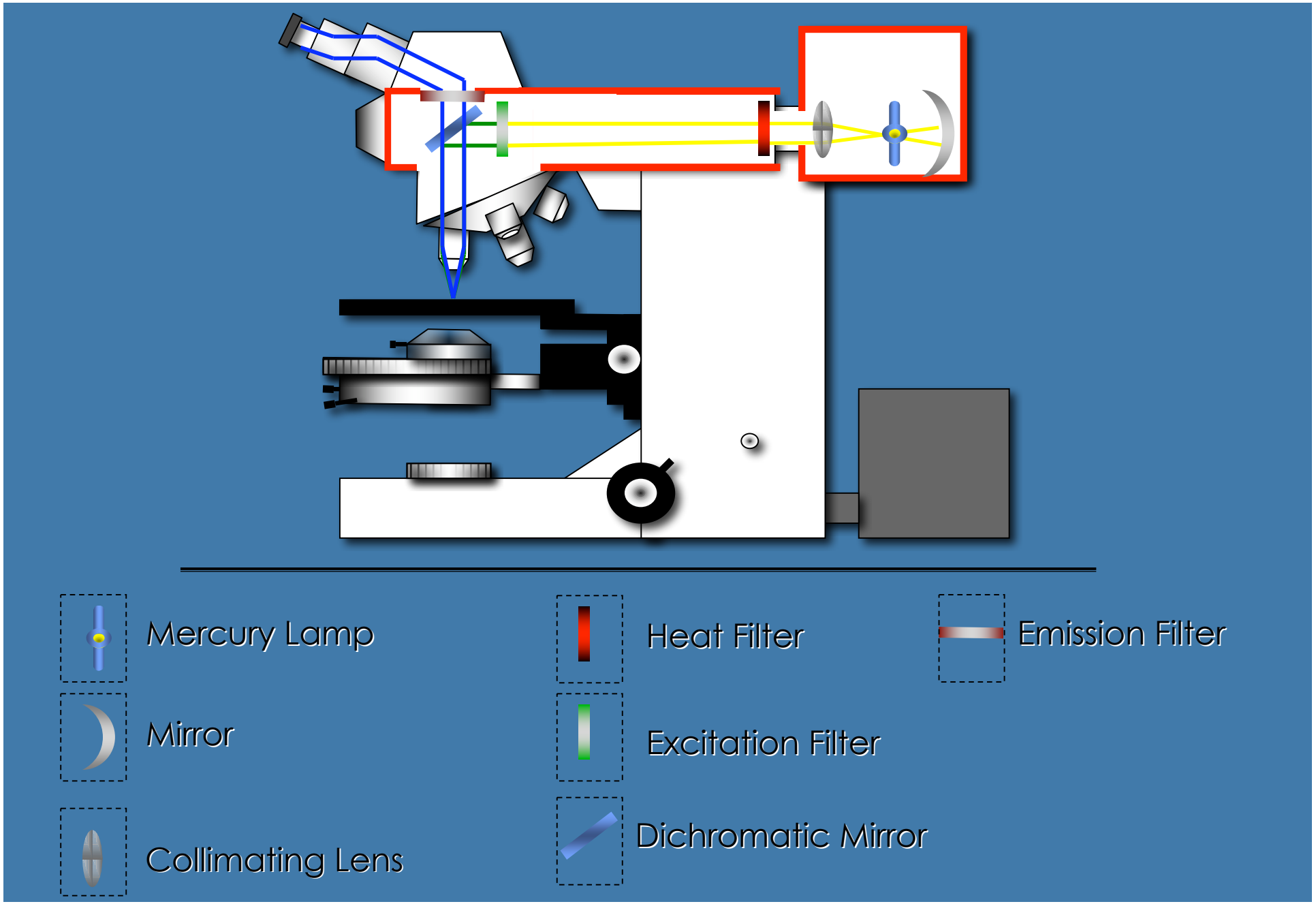


Spectrum of a Xenon Lamp

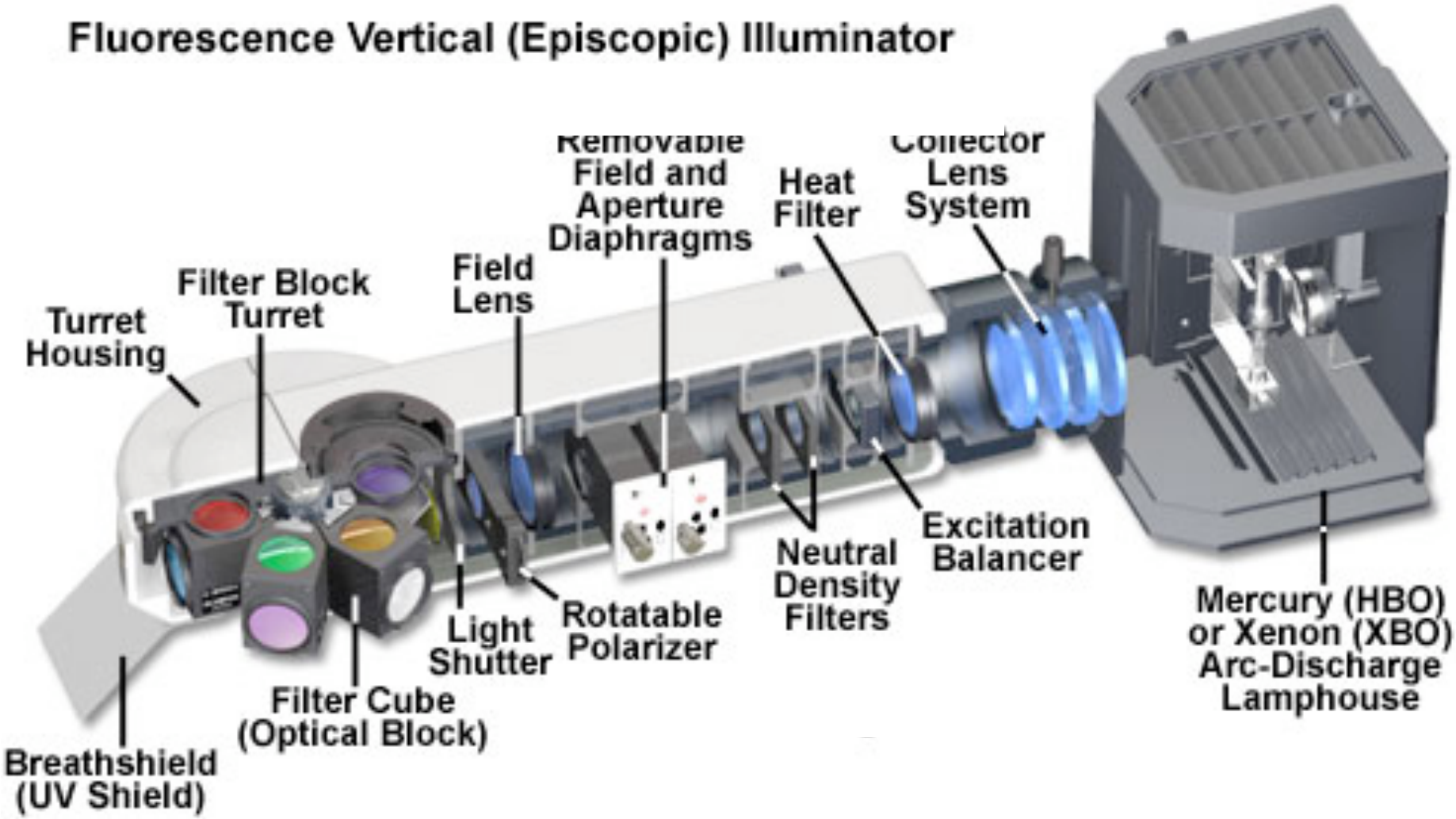


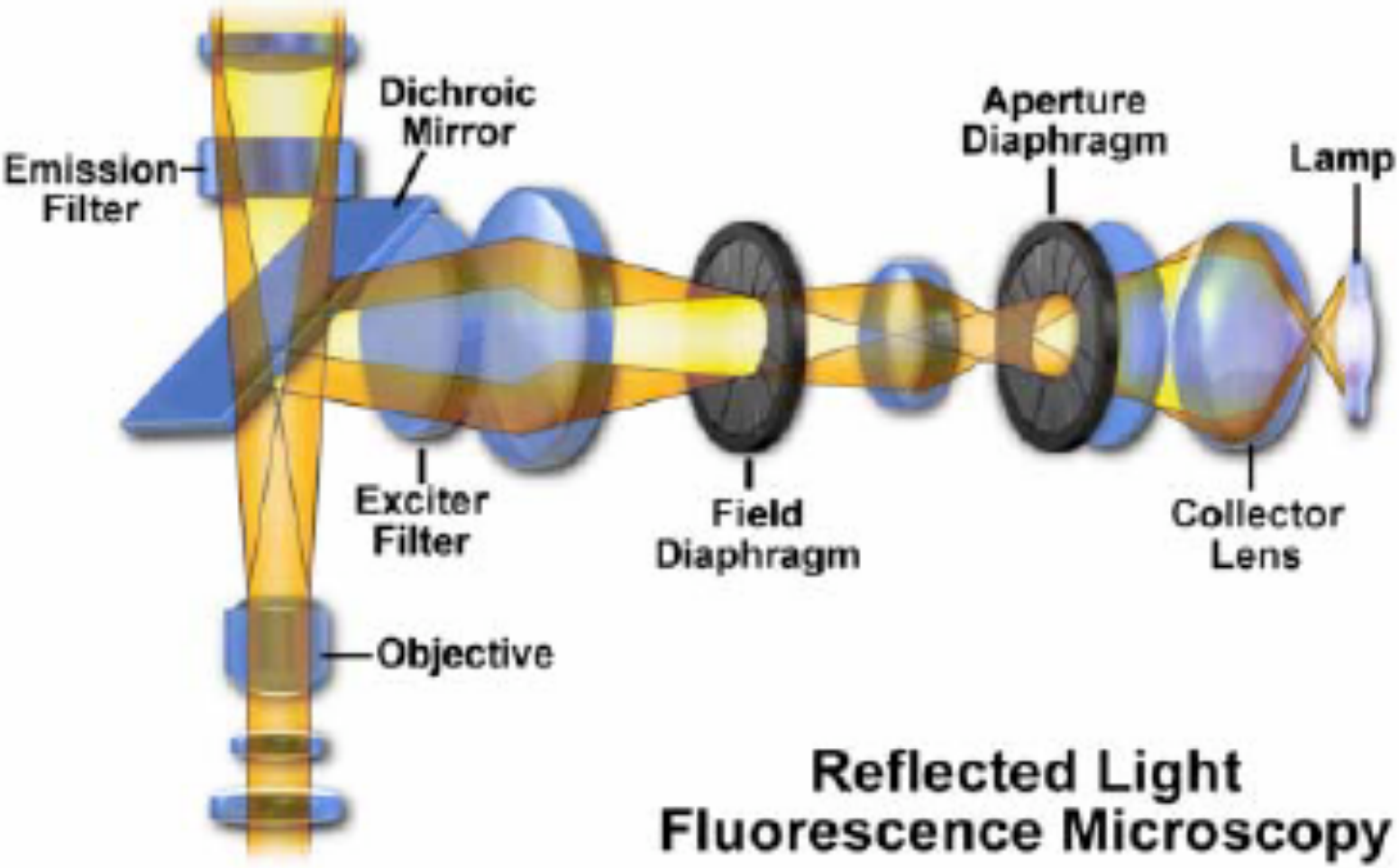
Light sources for fluorescence

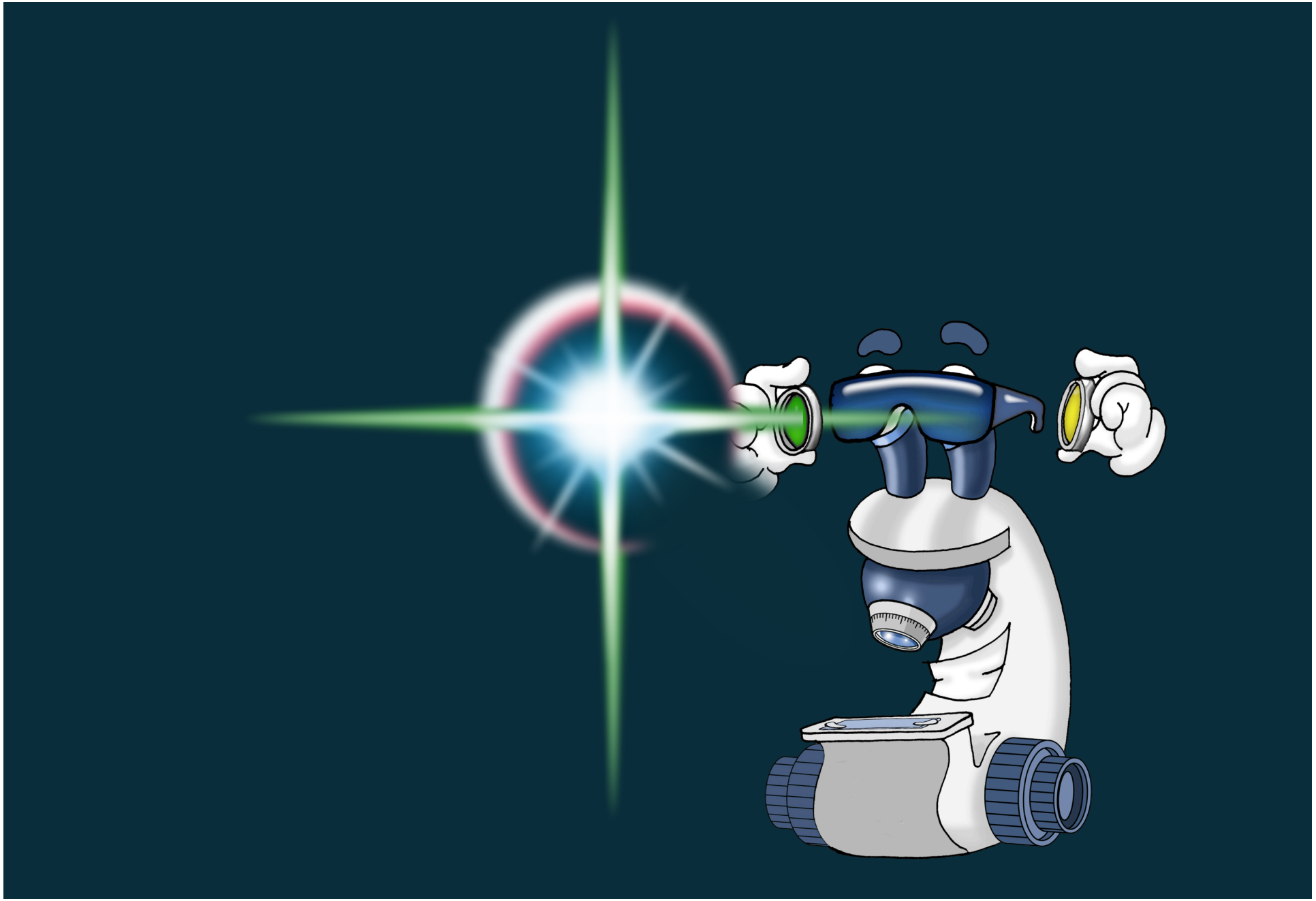
(Modified from: http://www.cairn-research.co.uk)

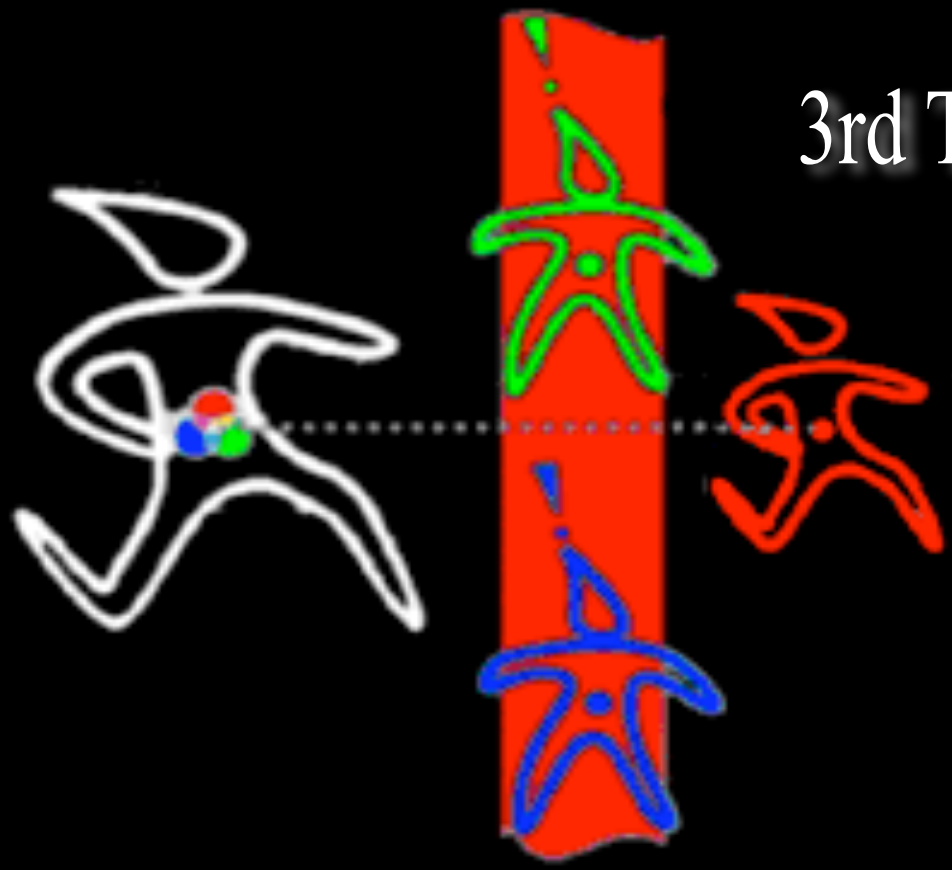

Mercury Lamp
Mirror
Collimating Lens
Heat Filter
Excitation Filter
Dichromatic Mirror
Emission Filter

Fluorescence Vertical (Episcopic) Illuminator

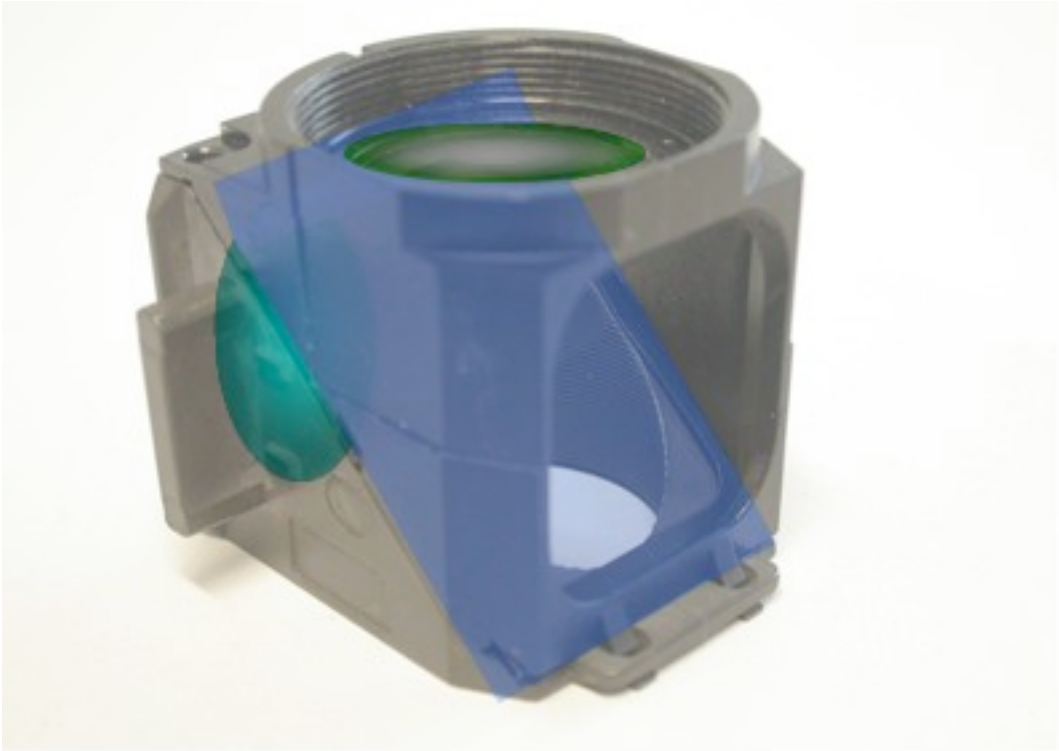



Dichroic
Mirror
Emission
Filter
Aperture
Diaphragm
Lamp
Exciter
Filter
Field
Diaphragm
Collector
Lens
Objective
Reflected Light
Fluorescence Microscopy

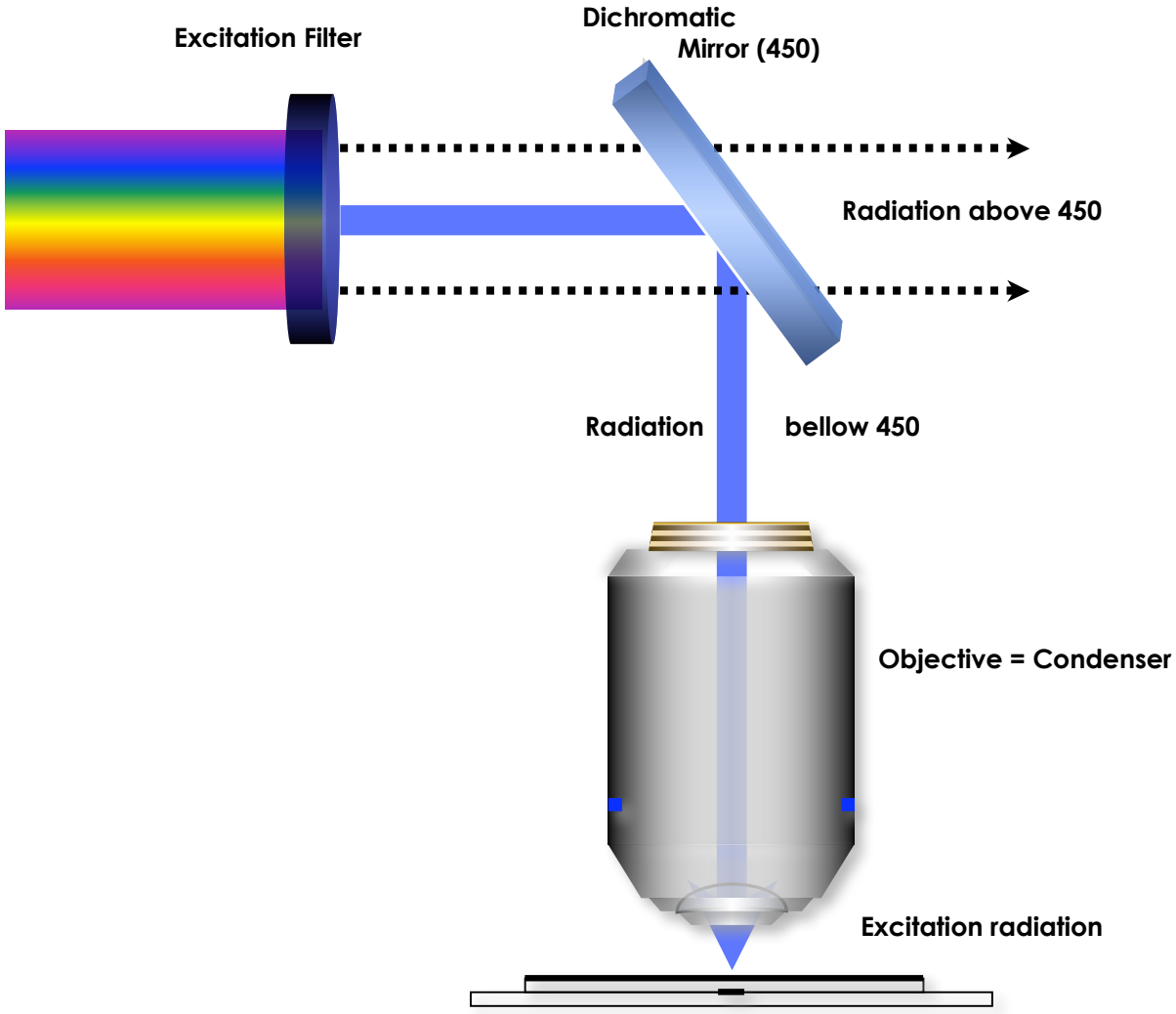


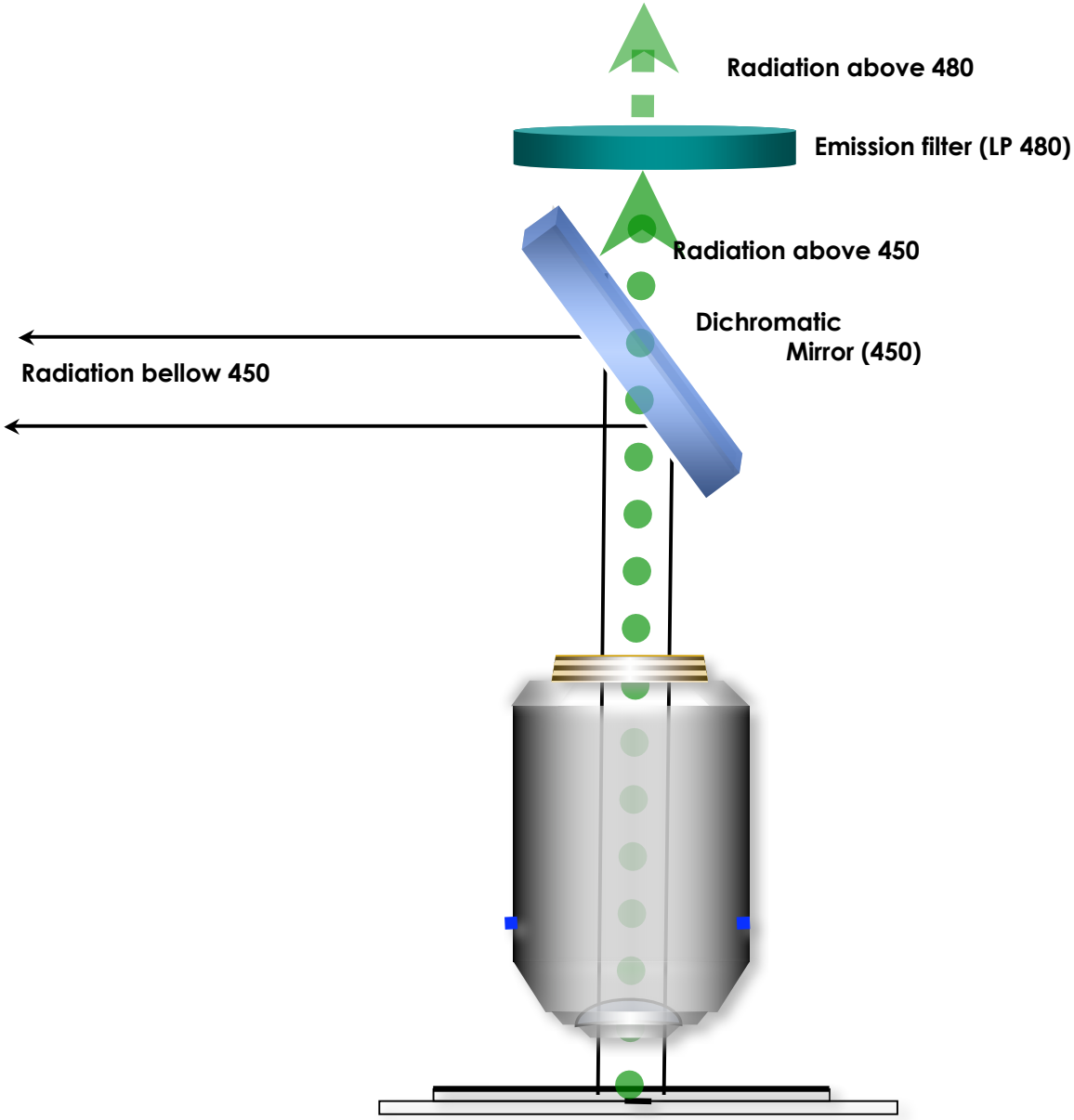


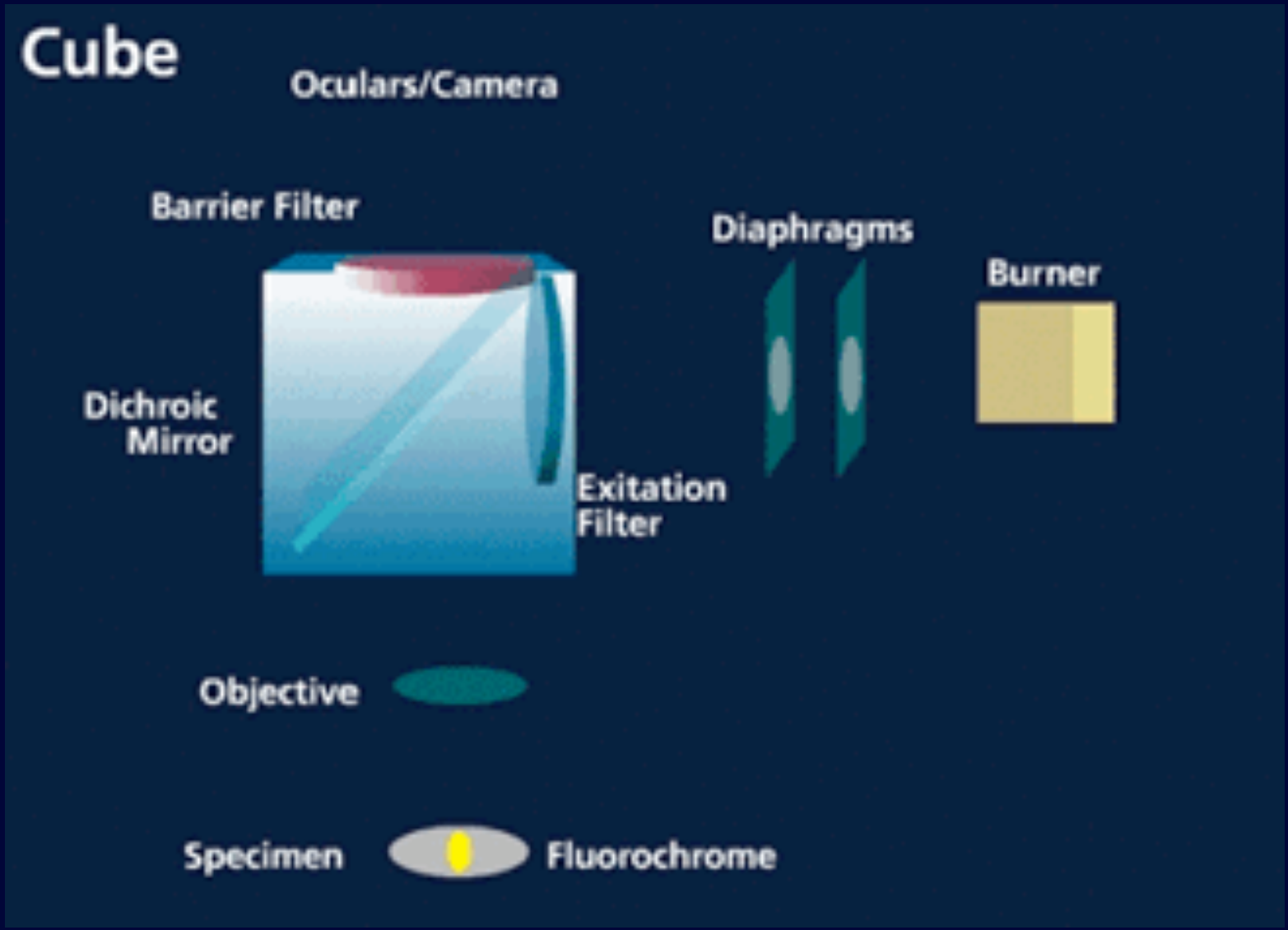
3rd TAKE HOME MESSAGE.....
KNOW YOUR FILTER SYSTEM!!!!



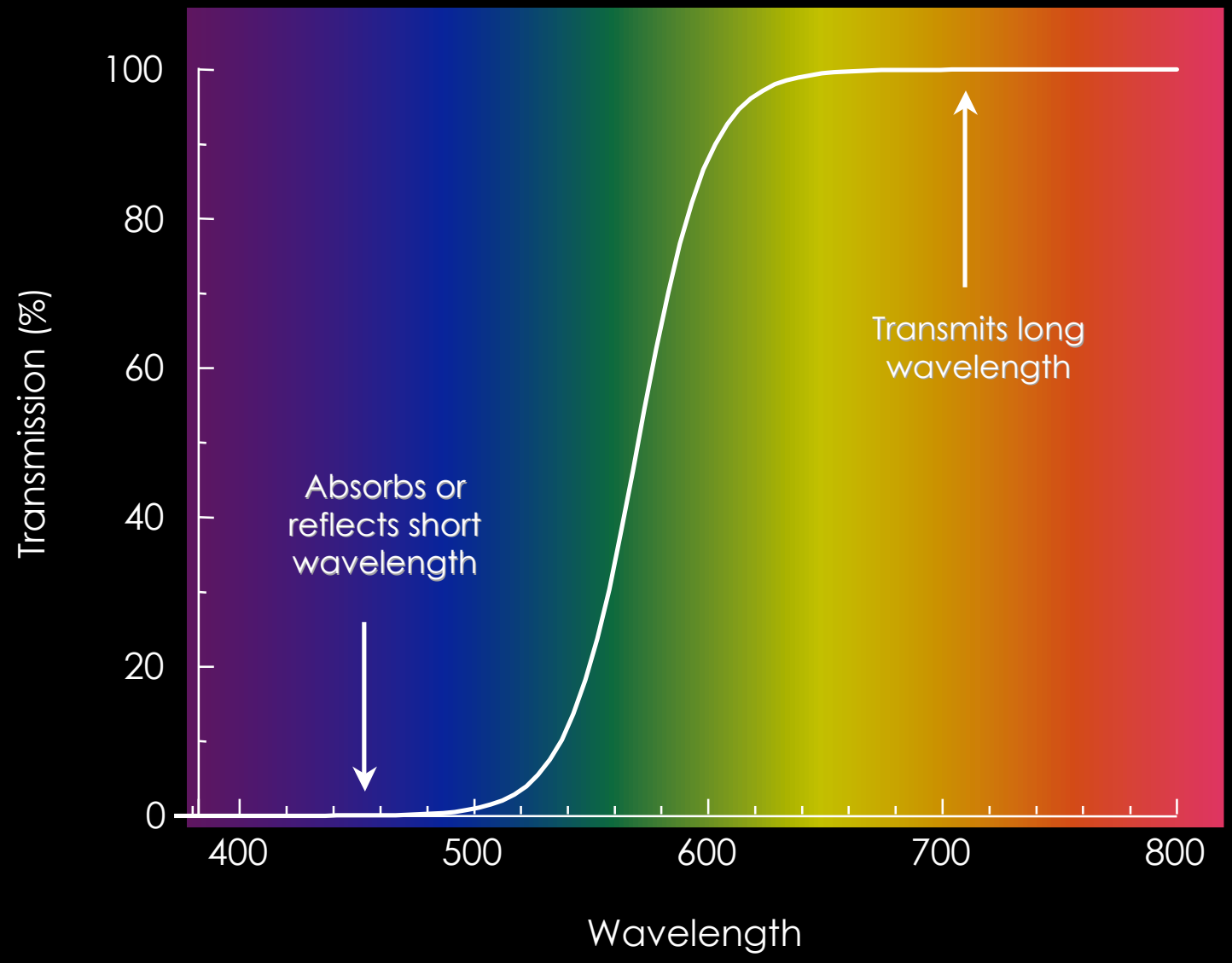
U-MNUA2
OLYMPUS
JAPAN


Excitation Filter
Dichromatic
Mirror (450)
Radiation above 450
Radiation
bellow 450
Objective = Condenser
Excitation radiation

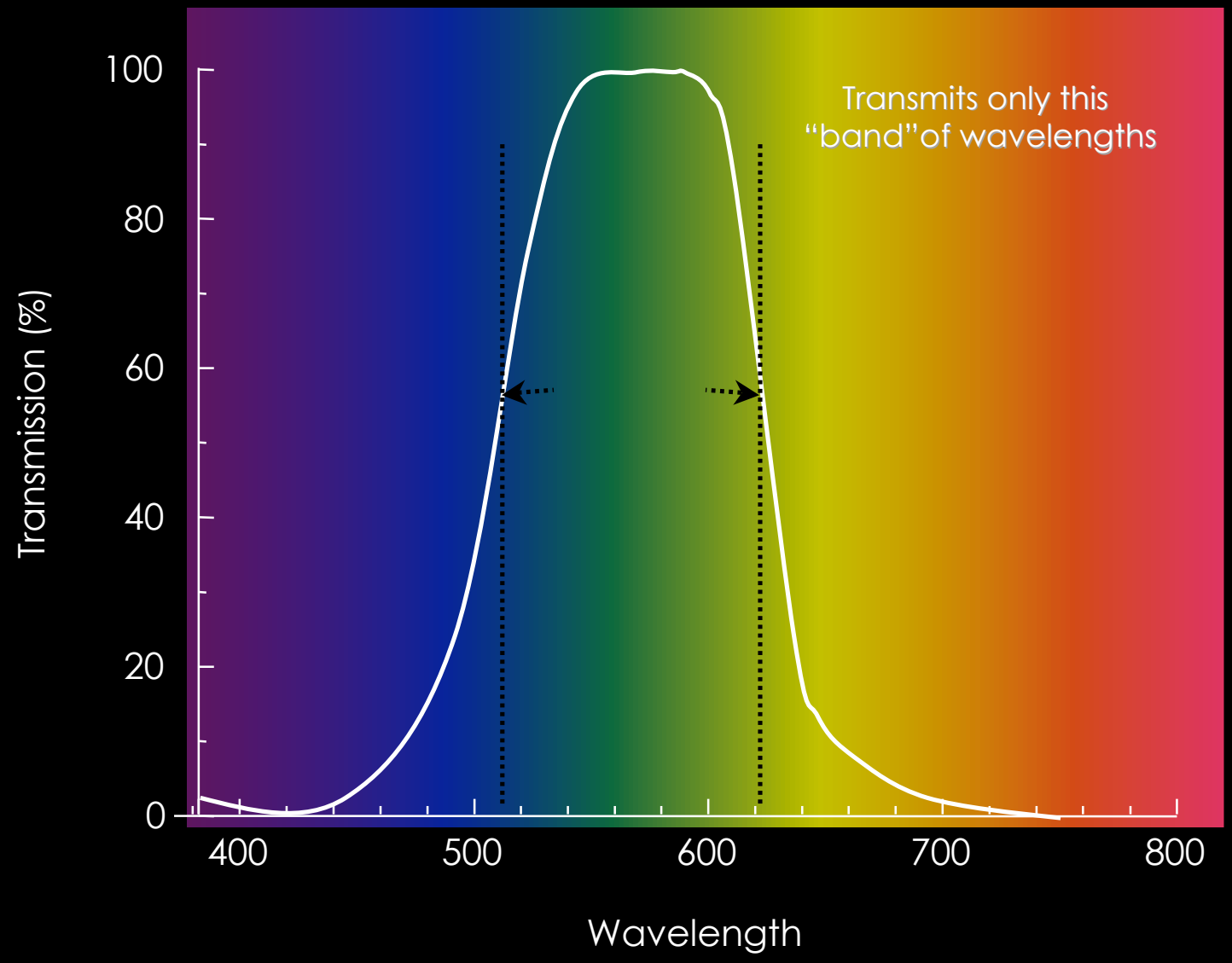

Radiation above 480
Emission filter (LP 480)
Radiation above 450
Dichromatic
Mirror (450)
Radiation bellow 450


Cube
Oculars/Camera
Barrier Filter
Diaphragms
Burner
Dichroic Mirror
Exitation Filter
Objective
Specimen
Fluorochrome

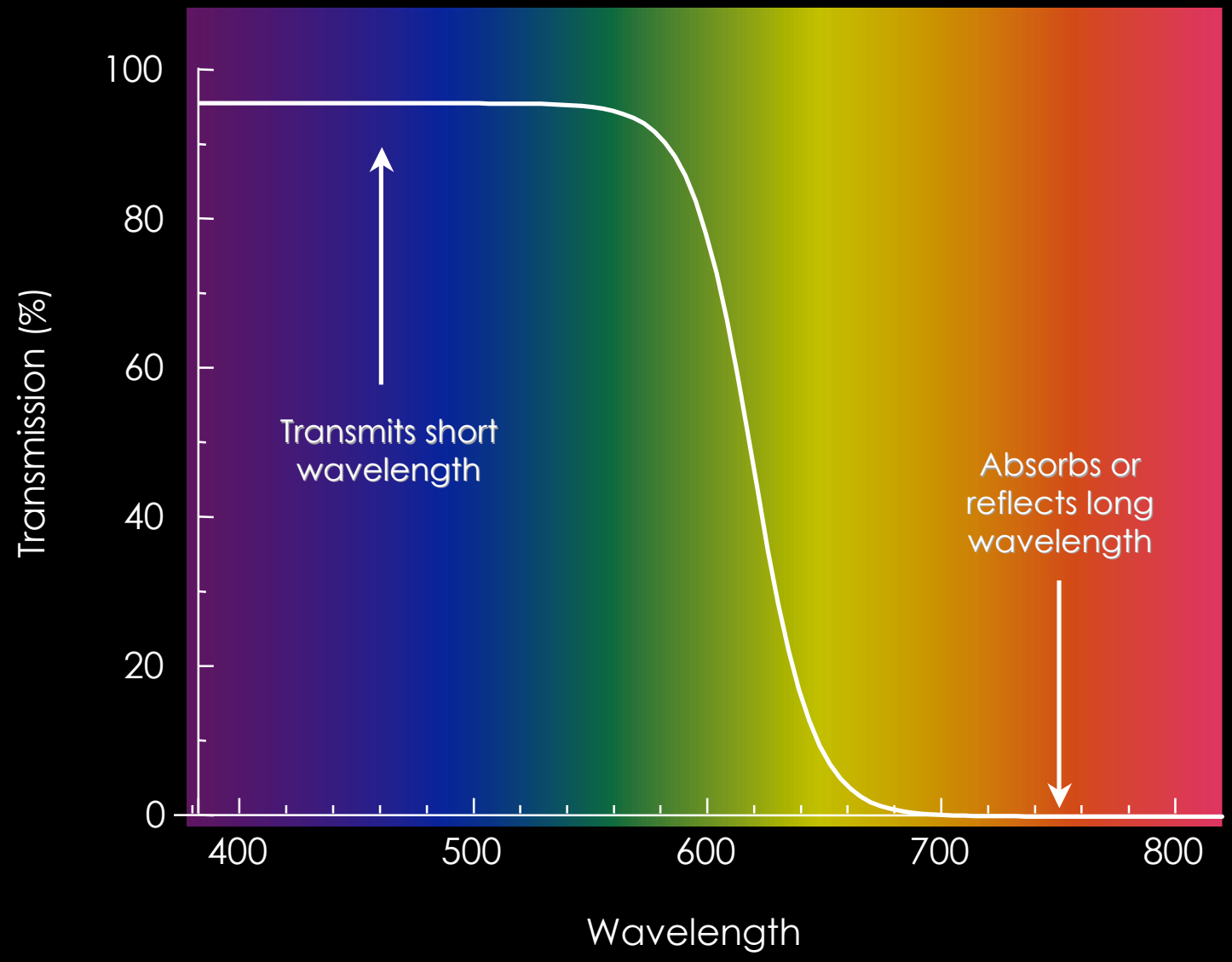
Long Wave Pass Filter (LWP)

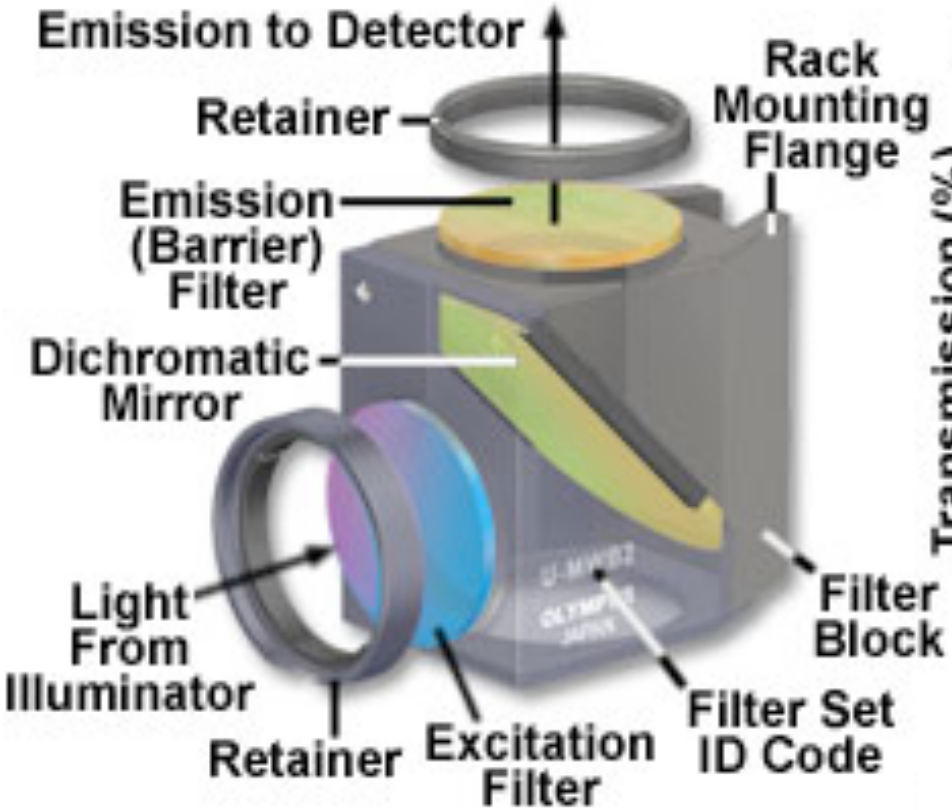


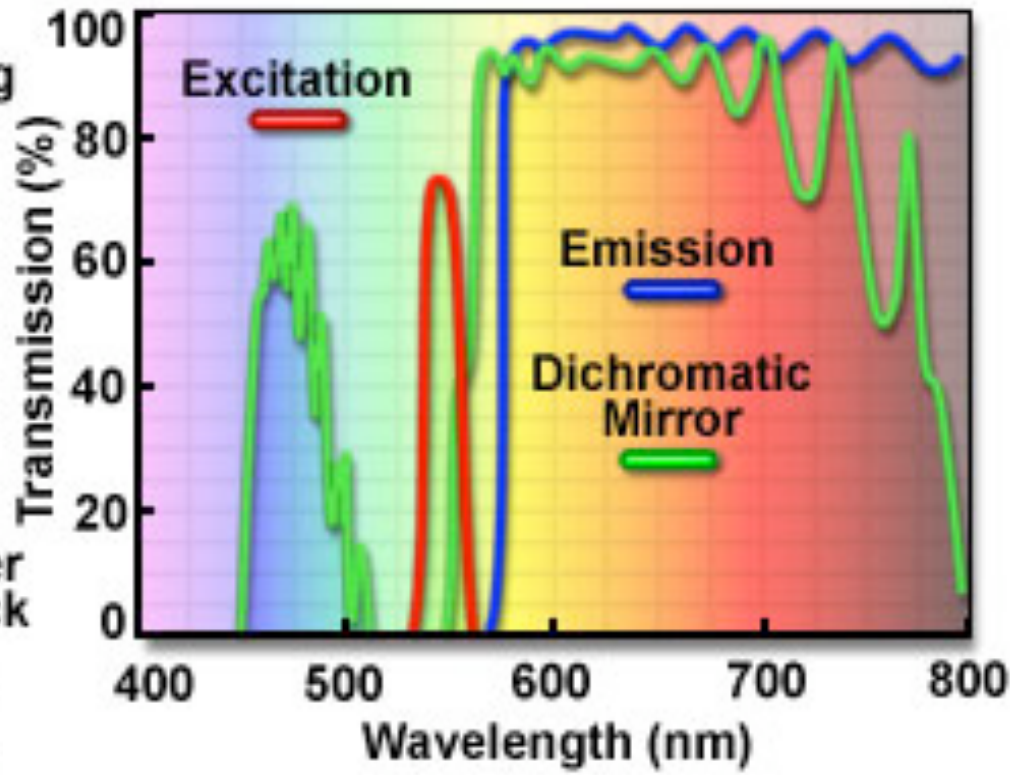
Bandpass Filter

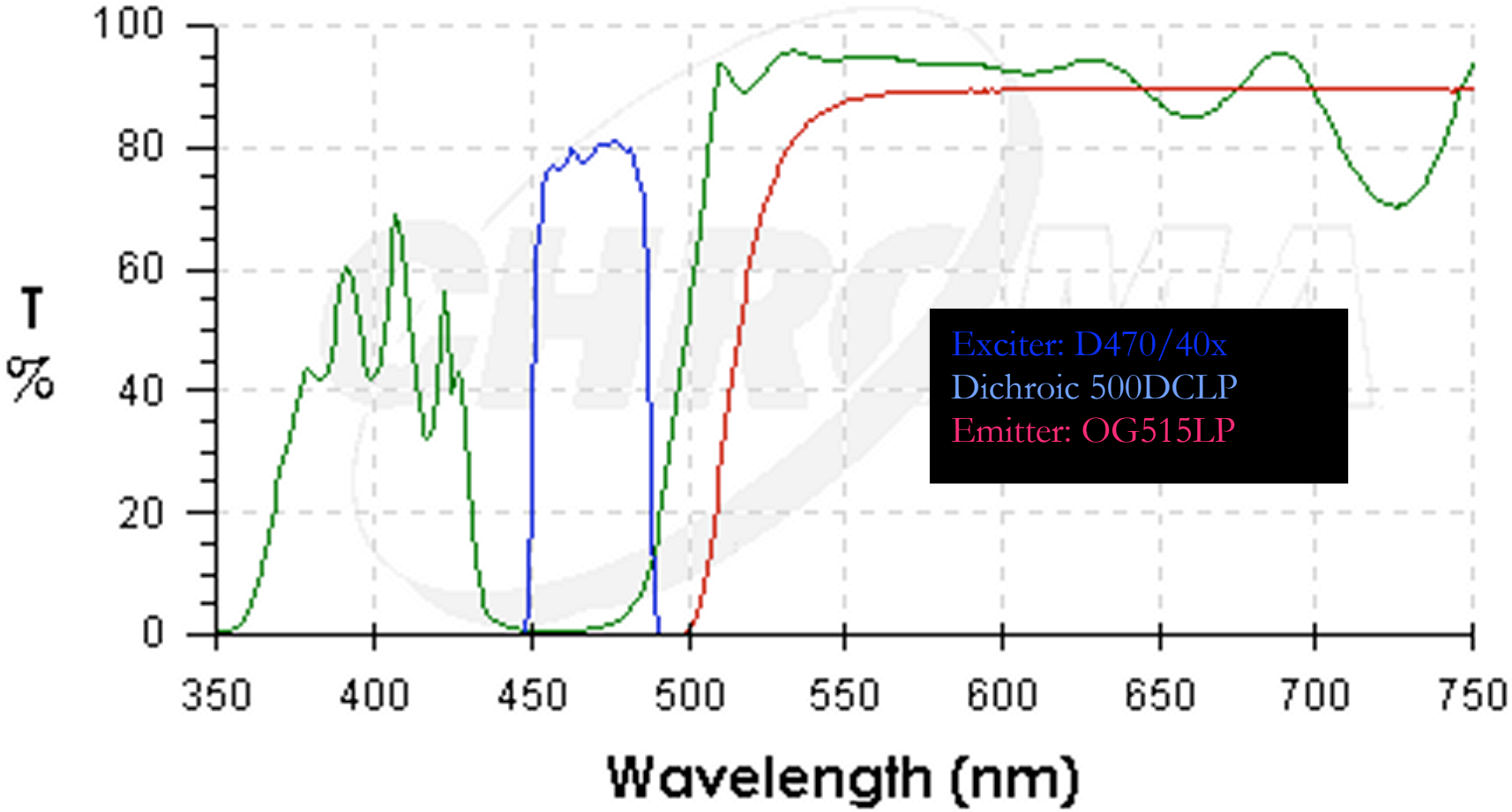


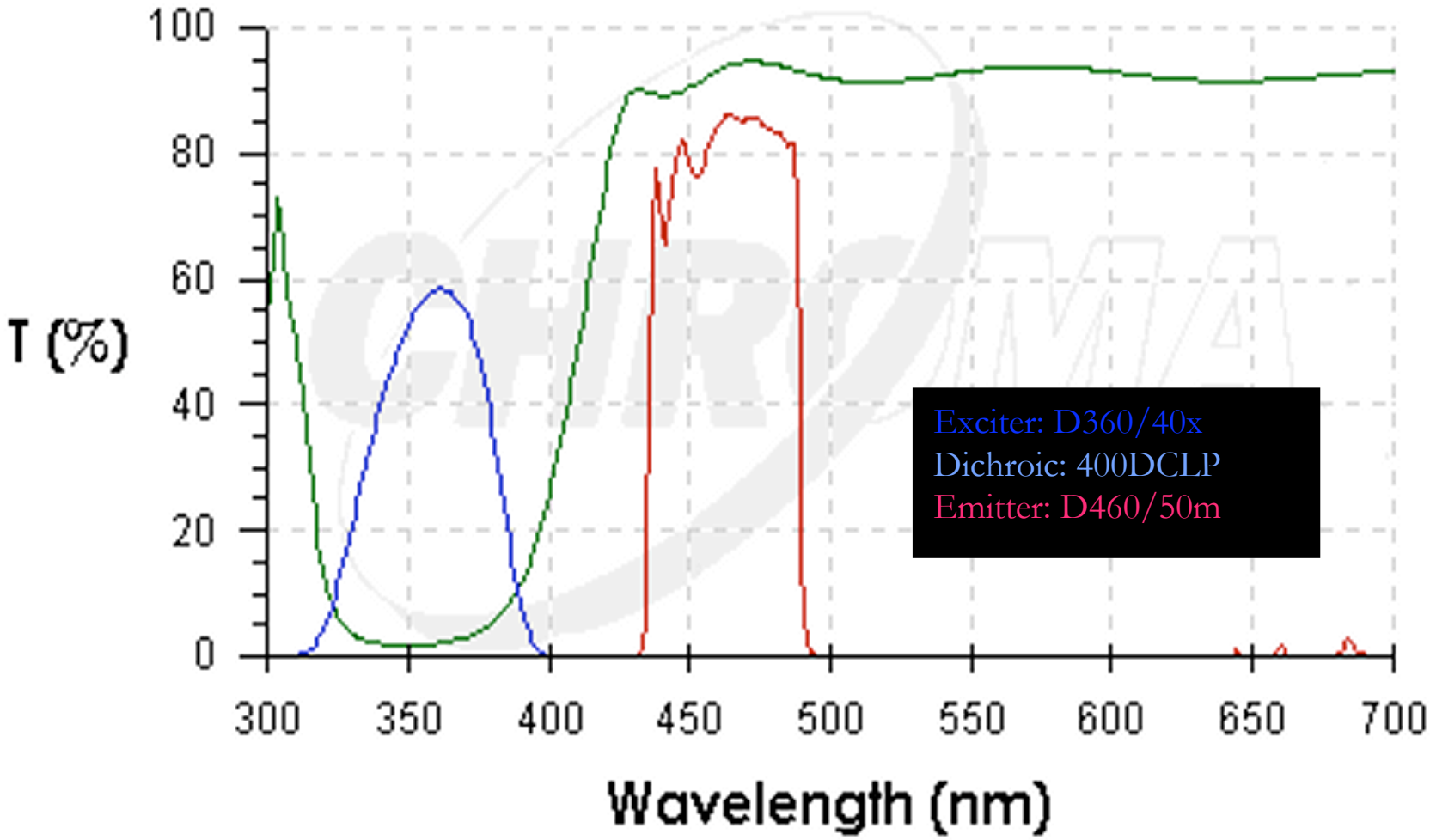
Short Wave Pass Filter (SWP)

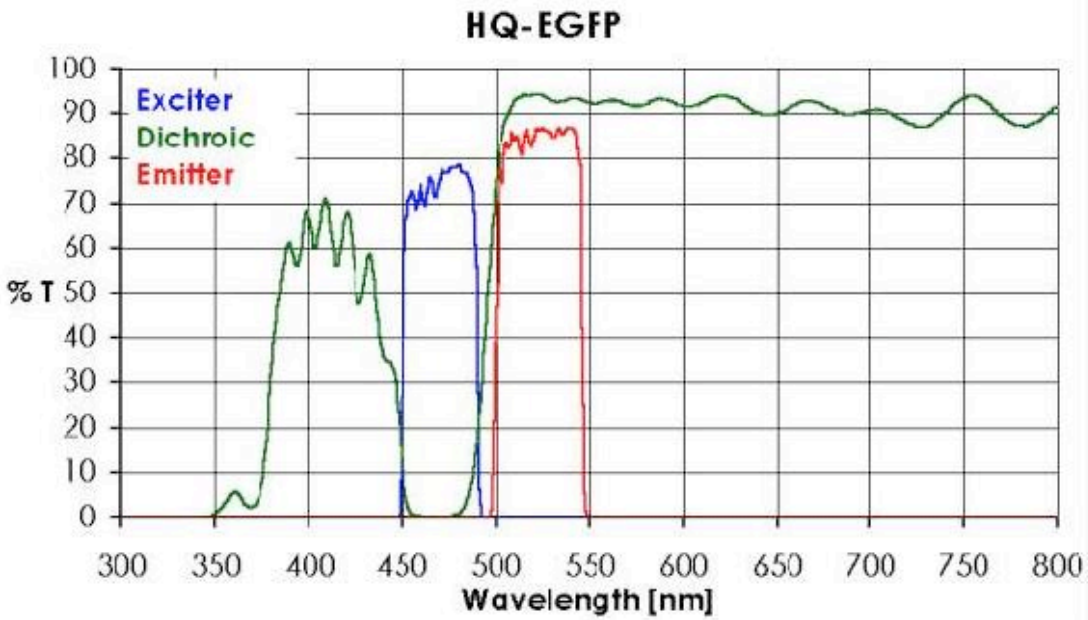



Emission to Detector
Retainer
Emission (Barrier) Filter
Dichromatic Mirror
Light From Illuminator
Retainer
Excitation Filter
Filter Set ID Code
Filter Block
Rack Mounting Flange

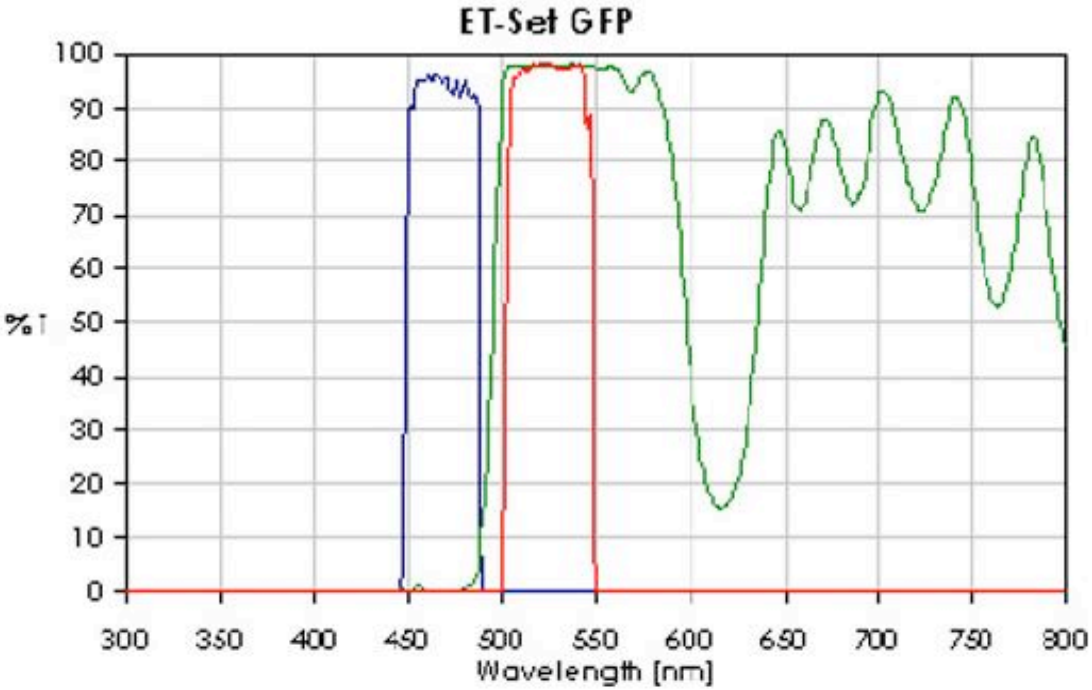

Excitation
Emission
Dichromatic Mirror
Transmission (%)
100
80
60
40
20
0
400
500
600
700
800
Wavelength (nm)


100
80
60
40
20
0
T
%
350
400
450
500
550
600
650
700
750
Wavelength (nm)
Exciter: D470/40x
Dichroic 500DCLP
Emitter: OG515LP


100
80
60
40
20
0
T (%)
300
350
400
450
500
550
600
650
700
Wavelength (nm)
Exciter: D360/40x
Dichroic: 400DCLP
Emitter: D460/50m

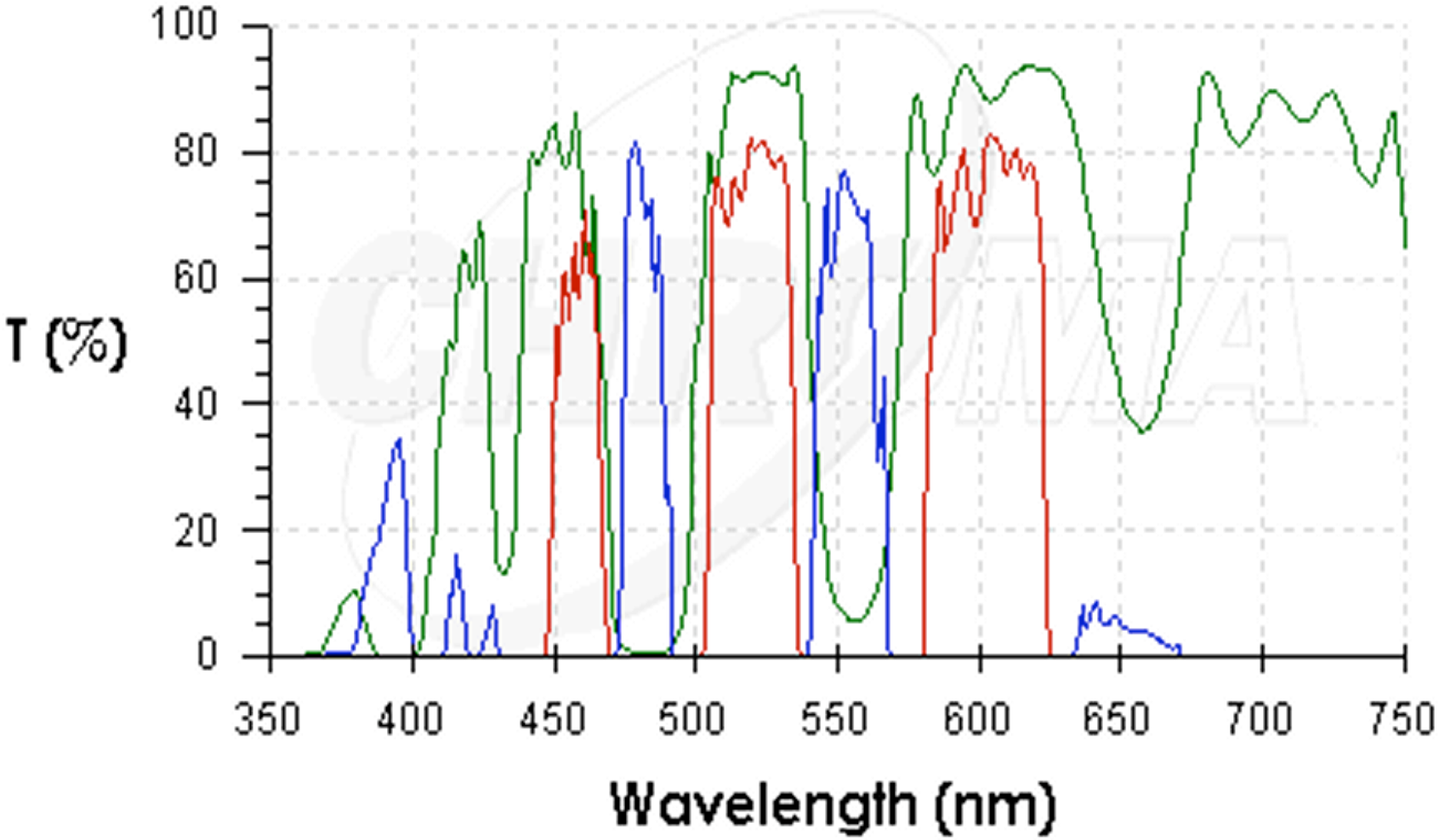


X: 470/40
M: 525/50
BS: 495 LP

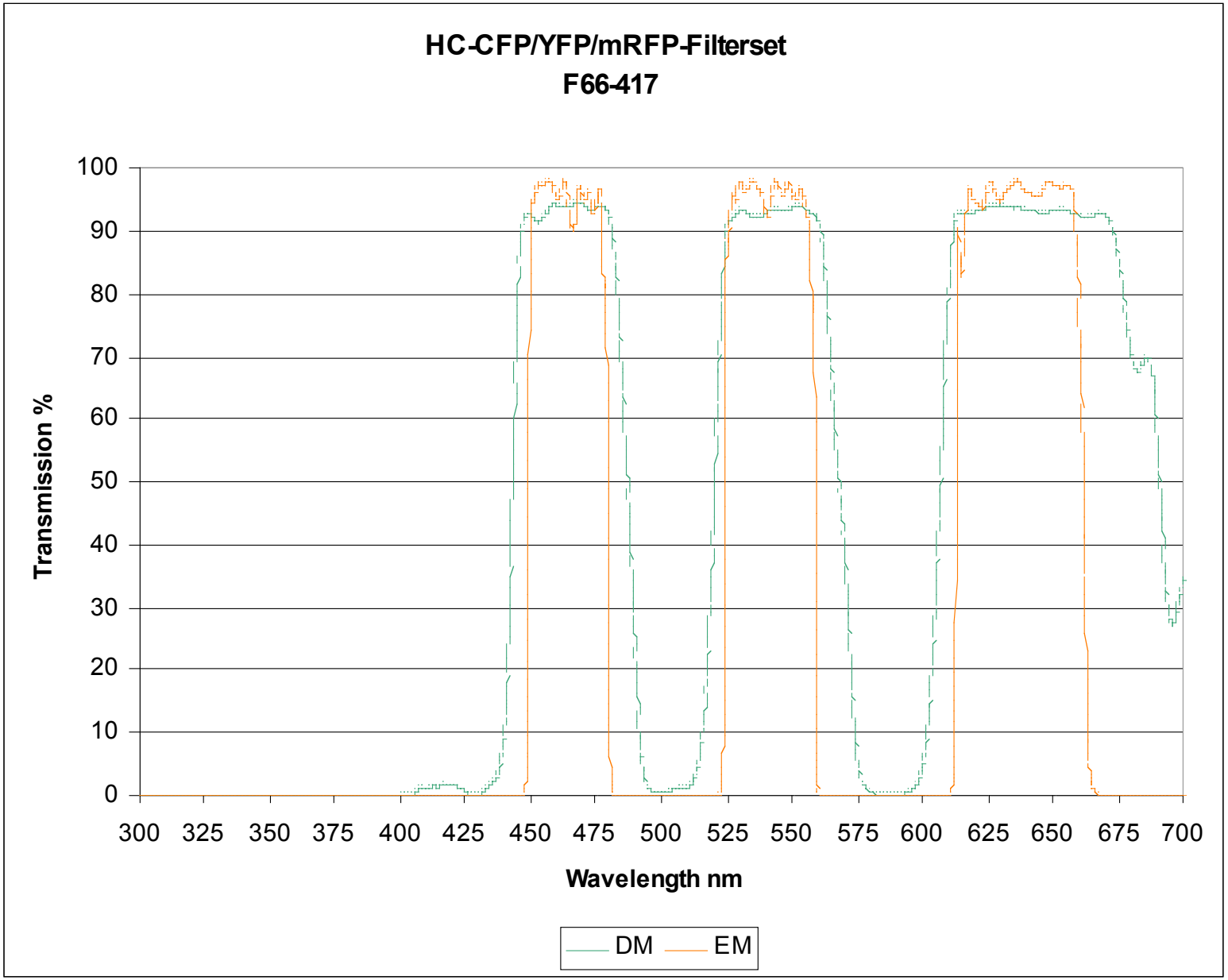


X: 470/40
M: 525/50
BS: 495 LP

(http://www.ahf.de)



Exciter: 61000v2x Dichroic 61000v2bs Emitter 61000v2m


HC-CFP/YFP/mRFP-Filterset
F66-417
Transmission %
100
90
80
70
60
50
40
30
20
10
0
300
325
350
375
400
425
450
475
500
525
550
575
600
625
650
675
700
Wavelength nm
DM
EM